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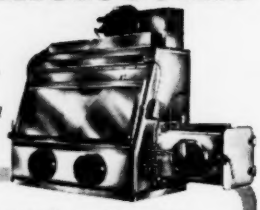

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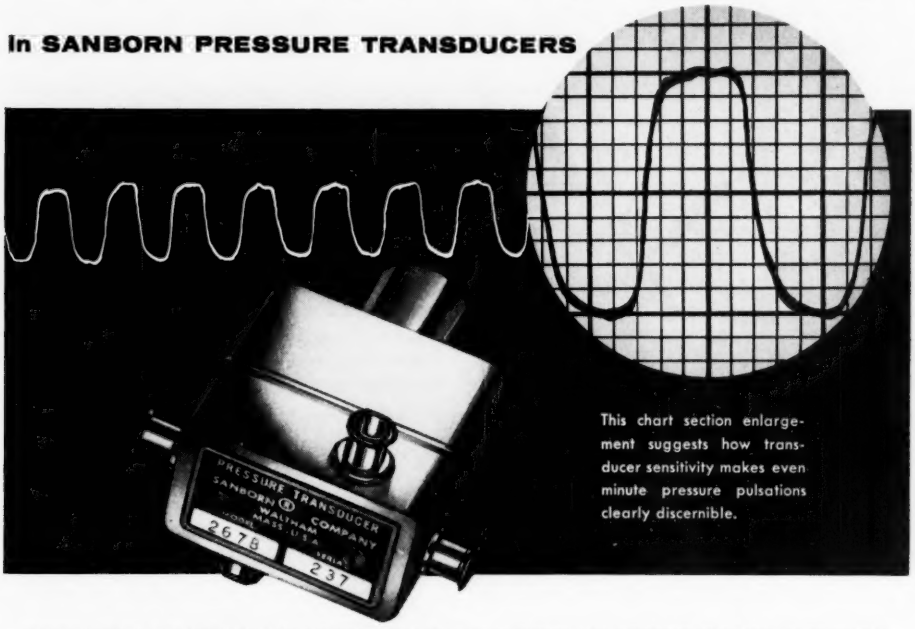

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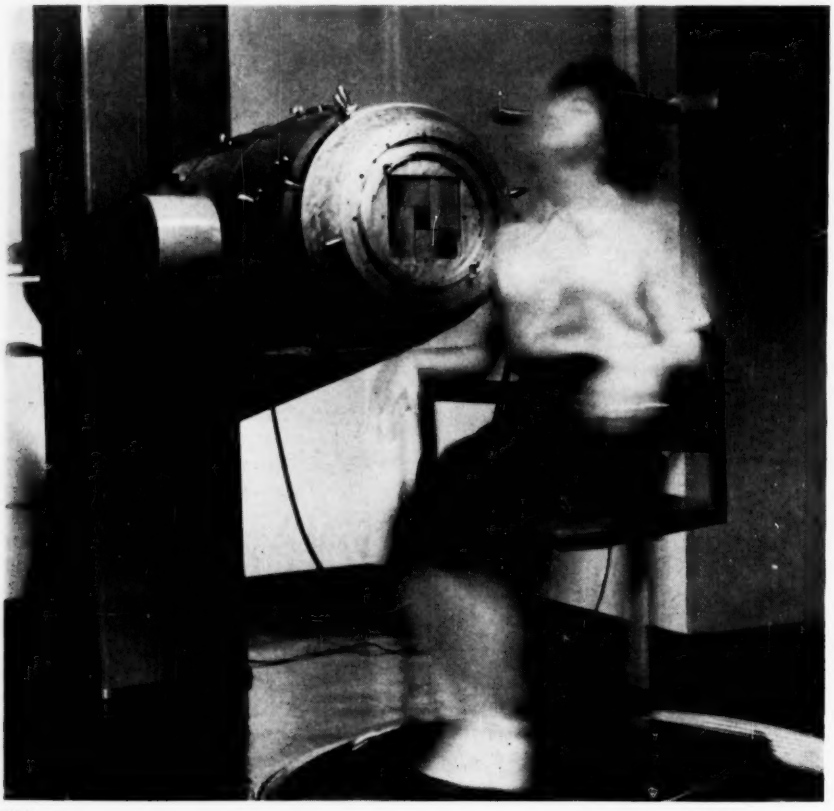

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PLASMA NONESTERIFIED FATTY ACIDS IN HYPERTHYROID STATES

By CLAYTON RICH, EDWIN L. BIERMAN * AND IRVING L. SCHWARTZ †

(From The Rockefeller Institute for Medical Research, New York, N. Y.)

(Submitted for publication August 25, 1958; accepted September 18, 1958)

Adipose tissue appears to be more than normally active in hyperthyroidism. The low fasting respiratory quotient and increased oxygen consumption (1, 2) as well as the predisposition to ketosis (3) of hyperthyroid subjects suggest that mobilization and oxidation of fat are accelerated. This inference is supported by the finding of an increased rate of turnover of fatty acids of thyroid-fed rats (4, 5).

Recent studies indicate that the nonesterified fatty acid fraction of plasma reflects the metabolic state of adipose tissue. The concentration of nonesterified fatty acids (NEFA) rises when energy is drawn from stored fat, as in fasting, uncontrolled diabetes and after epinephrine (6–8), and falls after administration of carbohydrate or insulin (6, 9). The fall after insulin administration has been related to diminished outflow of fatty acids from tissue stores (9–11). The sharp rise after epinephrine is probably due to action at the same site, since epinephrine causes an output of fatty acids from isolated adipose tissue (11). Thus the concentration of NEFA in plasma appears to be an index of the rate of mobilization of fat.

The present studies were undertaken to see if the altered fat metabolism of the hyperthyroid state would be reflected in changes of plasma NEFA concentration of patients with spontaneous hyperthyroidism and of euthyroid subjects treated with triiodothyronine during acute and long term experiments. In all subjects studied, the plasma NEFA concentration was found to be increased when the metabolic rate was elevated.

PROCEDURE

Group I. Measurements were made of plasma NEFA concentration (6) of plasma taken at about 9:30 a.m. (after an overnight fast) from nine patients with typical thyrotoxicosis. The diagnosis was established in each case by characteristic signs and symptoms, including elevated basal metabolic rate and thyroidal radioiodine uptake.

Group II. Six euthyroid subjects (four obese, one with sarcoidosis and one with a parathyroid adenoma) were tested as follows: Two control blood samples were taken in the morning (after an overnight fast which lasted until completion of the test) and then either 1 or 2 mg. of L-triiodothyronine (TRITH) or 15 mg. of L-triiodothyroacetic acid (TRIAC)[1] were given by vein and additional blood samples taken each hour for the next six hours. The basal metabolic rate was measured at the start of the test and after five and six hours. As a control, the procedure was carried out for each subject in the same way except that no medication was given. All samples were analyzed for NEFA, cholesterol (12) and glucose (13).

Group III. Seven euthyroid subjects hospitalized on the Infectious Disease Service were given TRITH as a therapeutic trial in the course of an unrelated study to be reported elsewhere.[2] These patients received a daily dose of 0.15 to 0.2 mg. of TRITH orally for 30 to 40 day periods alternating with similar periods during which no TRITH was given. Blood samples, taken each week after an overnight fast, were analyzed for cholesterol and NEFA. The basal metabolic rate was measured every two to four days.

RESULTS

Group I

Fasting NEFA values of nine hyperthyroid patients averaged 1,103 μEq. per L. with a standard deviation of 164 μEq. per L. This average was

* Present address: U.S.A. Medical Nutrition Laboratory, Fitzsimons Army Hospital, Denver, Colo.

† Present address: Medical Research Center, Brookhaven National Laboratory, Upton, Long Island, N. Y.

[1] L-Triiodothyronine (Cytomel®) and L-triiodothyroacetic acid were very generously supplied by Smith, Kline and French Laboratories, Philadelphia, Pa.

[2] These patients had moderately far advanced pulmonary tuberculosis. They were under continuous treatment with isoniazid (5 mg. per Kg. per day) and streptomycin (1 Gm. three times per week). The effect of TRITH on the course of their disease was under investigation because of observations in animals that thyroid hormone increases resistance to pulmonary tuberculosis. We are indebted to Drs. D. Böhme, S. Osterhout, R. W. Schaedler, H. Simon and J. G. Hirsch for the opportunity to carry out metabolic studies on these patients during the period when treatment with TRITH was being evaluated.

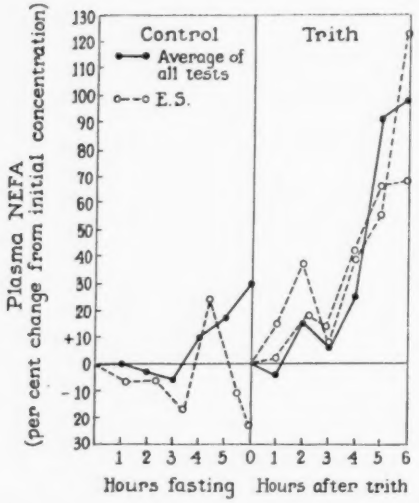

FIG. 1. COMPARISON OF THE CHANGES OF THE CONCENTRATION OF PLASMA OF NONESTERIFIED FATTY ACIDS (NEFA) IN PLASMA DURING A SIX HOUR PERIOD OF FASTING (CONTROL) AND DURING A SIMILAR PERIOD AFTER THE INTRAVENOUS ADMINISTRATION OF 1 OR 2 MG. L-TRIIODOTHYRONINE (TRITH)

Solid lines indicate the mean for all subjects studied. Dashed lines compare the individual responses of a single subject (E. S.) during one control and two TRITH tests.

significantly greater ("t" test, $p < 0.01$) than the normal fasting value of 572 ± 280 μEq. per L. (8).

Group II

A total of 18 tests were made, 7 control, 7 in which 1 or 2 mg. of TRITH and 4 in which 15 mg. of TRIAC was given. NEFA concentration, but not the basal metabolic rate, usually rose slightly during the control test. In contrast, during the six hours after intravenous administration of TRITH, there was a sharp increase of NEFA concentration in every subject to reach values averaging 592 ± 254 μEq. per L. greater than the original values (Figure 1). This increase was significantly greater ($p < 0.01$) than that observed during the control test when no TRITH was given (Table I). After administration of TRITH, oxygen consumption rose significantly ($p < 0.01$) above control values, but no significant changes of glucose or cholesterol concentrations were observed (Table I). From about the eighth through the thirty-sixth hour after TRITH most subjects showed one or more of the following signs and symptoms of thyroid effect: tachycardia, nervousness, headache, rise in systolic pressure and, occasionally, rise in body temperature (less than 1° C.).

Only one of the four subjects given 15 mg. TRIAC had any effect from this dose. The subject who did respond showed the same changes which were observed after TRITH: a sharp rise in NEFA and basal metabolic rate, but no alteration of either cholesterol or glucose concentration.

Group III

During the first week of treatment, all subjects given 0.15 or 0.20 mg. TRITH per day developed clinical signs of thyroid effects, with marked elevation of metabolic rate, fall of serum cholesterol and rise of NEFA (Figure 2). These effects disappeared during the first 10 days after TRITH was discontinued and returned when it was given again. (A typical response is shown in Figure 3.) During the control period the mean of the plasma NEFA concentrations of all subjects in this group

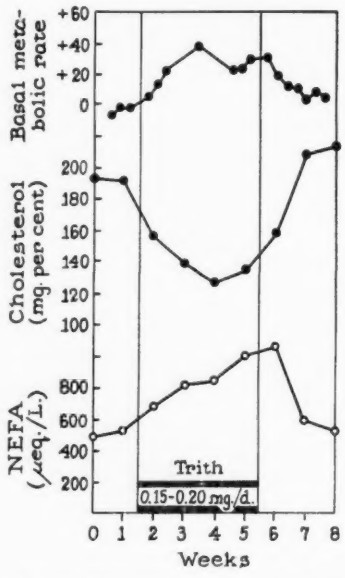

FIG. 2. MEAN CHANGES OF PLASMA, CHOLESTEROL AND NEFA CONCENTRATIONS AND OF BASAL METABOLIC RATES OF EUTHYROID SUBJECTS TREATED WITH L-TRIIODOTHYRONINE (TRITH) FOR ONE MONTH

TABLE I

Comparison of changes produced during a six hour fast (control) and during a similar period after 1 or 2 mg. triiodothyronine (TRITH) by vein

	Test	Mean value ± S. D.* for all subjects studied		Mean difference between original and final values	Significance of difference between control and TRITH tests (p)
		Start of test	End of test		
Plasma NEFA *μEq./L.*	Control	855 ± 243	873 ± 128	+ 18	<0.01
	TRITH	768 ± 214	1,360 ± 306	+592	
Oxygen consumption *ml./min.*	Control	245 ± 24	241 ± 17	− 4	<0.01
	TRITH	231 ± 27	275 ± 41	+44	
Plasma glucose *mg./100 ml.*	Control	70.8 ± 8.4	74.3 ± 10.0	−3.7	>0.5
	TRITH	77.2 ± 6.7	77.0 ± 5.9	−0.2	
Serum cholesterol *mg./100 ml.*	Control	161 ± 24	161 ± 25	0	>0.5
	TRITH	189 ± 30	185 ± 34	−4	

* Standard deviation.

was 520 μEq. per L., with a standard deviation of 145 μEq. per L. By the fourth week of treatment (Figure 2) this value had risen significantly ($p < 0.01$) to 1,020 ± 316 μEq. per L., a level comparable to that already described for subjects with spontaneous hyperthyroidism.

DISCUSSION

Glycogen depletion and rapid carbohydrate oxidation (3, 4, 14) of hyperthyroid patients are such that, despite accelerated gluconeogenesis (3, 4), these subjects are particularly dependent upon fat as the major energy source. Within six hours of intravenous administration of TRITH, plasma NEFA rose to values in the range observed in naturally occurring or induced hyperthyroidism. This change appeared at the same time that accelerated heat production was first evident, in the absence of any alteration of blood glucose or cholesterol concentration and long before the fall of cholesterol or other blood lipids is known to occur (15). This suggests that the plasma NEFA rise is directly associated with accelerated mobilization and oxidation of fat necessary to support increased caloric output.

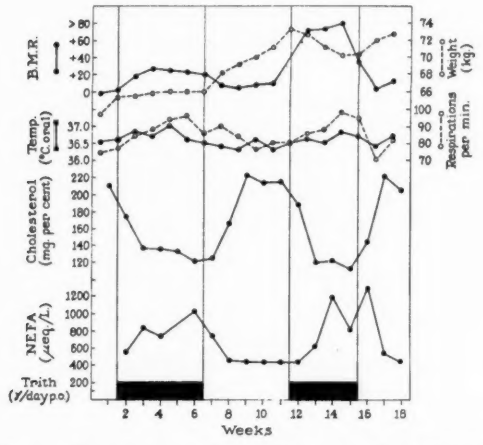

FIG. 3. RESPONSE OF A SINGLE SUBJECT TO TWO PERIODS OF TREATMENT WITH L-TRIIODOTHYRONINE (TRITH)

SUMMARY

1. Plasma nonesterified fatty acid (NEFA) concentration was found to be significantly increased when the basal metabolic rate was elevated in subjects with spontaneous and induced hyperthyroidism.

2. Euthyroid subjects showed an increased NEFA concentration within six hours after the intravenous administration of L-triiodothyronine, when accelerated oxygen consumption was first observed but before other signs or symptoms of hyperthyroidism appeared.

3. These results suggest that the increased plasma NEFA concentration reflects the accelerated mobilization of fat necessary to support the greater metabolic requirement of hyperthyroid subjects.

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REACTION OF I-131 TRACE LABELED HUMAN ANTI-Rh_0(D) WITH RED CELLS * †

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The reaction between red cell antigens and their corresponding isoantibodies is believed to occur in two distinct stages (1–3). The first stage involves the binding of the antibody to the antigen on the red cell stroma. It is generally conceded that this process represents a specific immunochemical reaction commonly referred to as "sensitization" of the red cell. The second stage which follows this reaction results in the visible agglutination of the red cell mass. The agglutination of red cells is a nonspecific process which can be significantly influenced by the nature of the suspending medium or by changes in the cell membrane. Both of these processes, "sensitization" and agglutination, are evident in the case of complete or saline active isoantibodies. With incomplete or blocking antibodies (4, 5), however, only the first stage is evident so that the isoantibody sensitizes the red cell but the reaction does not proceed to visible agglutination.

Laboratory detection of these antibodies consequently depends on enhancing the nonspecific agglutination stage. Enhancement of agglutination is commonly achieved through the use of three techniques: the use of large anisometric molecules in the cell suspending medium (6), enzyme digestion of the red cell stroma (7, 8), or use of the anti-globulin reaction (9). The primary or specific reaction between the red cell and its isoantibody, therefore, cannot be observed directly. Recognition of this reaction depends on an inference derived from the nonspecific reaction of agglutination.

The use of an I-131 labeled isoantibody (10) offers a means of studying directly the reaction of an incomplete isoantibody with red cells. Boursnell, Coombs and Rizk (11) appreciated the possibilities offered by the I-131 protein label and tagged human anti-Rh_0(D) serum. They were able to use the labeled isoantibody to estimate the number of Rh_0(D) sites and the Paul-Bunnell sites on the red cell, and more recently the antigen sites on the rabbit red cell (12). Melcher, Steinfeld and Reed (13), using both I-131 sheep red cell agglutinating rabbit serum and an I-131 incomplete red cell antibody, obtained from a patient with acquired hemolytic anemia, were able to show by zone electrophoresis that the red cell isoantibody activity was associated with the gamma globulin fraction.

Although these studies established the usefulness of I-131 labeled red cell isoantibodies, they were severely limited by the binding of nonspecific I-131 labeled proteins to the red cells. The problem of nonspecific binding of labeled proteins to red cells was re-examined in this report. The studies to be presented have shown that by the use of alcohol fractionation and elution techniques an I-131 trace labeled anti-Rh_0(D) can be obtained that reacts specifically with Rh_0(D) positive red cells.

MATERIALS AND METHODS

Fractionation of anti-Rh_0(D) plasma. High titered anti-Rh_0(D) plasma was obtained from a 30 year old white housewife who had been immunized to Rh_0(D) by eight pregnancies. The last nonviable delivery occurred in February, 1955, at which time she had both Fallopian tubes ligated. Her phenotype was A_2, rh(cde), and her serum contained an incomplete anti-Rh_0(D) with a titer of 64. She was injected intravenously with 1 ml. of type A_1, Rh_0(cDe) blood and phlebotomized 13 days later (14-16). The blood was collected by means of an ion-exchange plastic donor set that contained Dowex 50®. At this time her serum incomplete anti-Rh_0(D) titer was 8,192. The specificity of the antibody was anti-Rh_0(D) as determined by the anti-globulin reaction and a panel of known red cells (Panocell®, Knickerbocker, New York, N. Y.).

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† Presented in part at the annual meeting of the American Association of Blood Banks, November 4–6, 1957, Chicago, Ill.

The calcium-free plasma was fractionated by a modification [1] of the Cohn ethanol method 10 procedure (17). In this modification Fraction III 2 is first removed by absorption onto $BaSO_4$, followed by precipitation of Fraction I at pH 6.8, $\Gamma/2 = 0.08$, 6 per cent ethanol at $-2°$ C. Fraction II + III 0,1,3 is then precipitated at pH 5.8, $\Gamma/2 = 0.04$, 19 per cent ethanol at $-5°$ C. Fraction II was then extracted from the II + III 0,1,3 paste with an acetate buffer at pH 4.9 containing 0.6 M glycine, $\Gamma/2 = 0.002$, 14 per cent ethanol at $-5°$ C. The remaining precipitate (Fraction III 0,1,3) was resuspended in 0.15 M phosphate buffer, pH 7.5. This fraction was subsequently designated A-18. In the other preparation, A-15, Fraction III-0 was extracted from the precipitated Fraction III 0,1,3 at pH 6.8, 0.6 M glycine, $\Gamma/2 = 0.005$, 15 per cent ethanol at $-5°$ C. The remaining paste (Fraction III 1,3) was dissolved in 0.15 M phosphate buffer, pH 7.5. Both preparations, A-18 and A-15, after fractionation were dialyzed against 0.15 M phosphate buffer, pH 7.5, preparatory to iodination.

The titer of anti-Rh_0(D) of the calcium-free plasma was 2,048 before fractionation. After fractionation and dialysis the anti-Rh_0(D) titer of A-18 was 1,024 and that of A-15 was 256. Anti-Rh_0(D) titers were determined using the method recommended by Dunsford and Bowley (1). Preparation A-15 contained 7.11 per cent and preparation A-18, 16.8 per cent of the original plasma nitrogen.

Iodination. The isoantibody-containing fractions were trace labeled with I-131 [2] by the tri-iodide method (I_3^-) at pH 7.5 in the presence of 0.15 M phosphate buffer (18, 19). The total nitrogen employed for iodination was 72 mg. for A-15 and 150 mg. for a A-18 at a concentration of 1.45 and 3.01 mg. nitrogen per ml., respectively. Unbound iodide-131 was removed by dialysis at 4° C. with a minimum of 20 L. of 0.15 M phosphate buffer at pH 7.5 containing 1 : 10,000 merthiolate over a 36 to 72 hour period. At the completion of dialysis the I-131 isoantibody preparation was clarified by centrifugation in a Spinco Model L Ultracentrifuge at 20,000 rpm for 10 minutes, maximum G 46,900.

Nonprotein-bound I-131 was determined by precipitating the I-131 proteins with 10 per cent cold trichloracetic acid in the presence of carrier iodide and carrier protein (human serum). The total quantity of iodine bound to the protein was calculated as described previously (18) using a molecular weight of 160,000. The properties of the two isoantibody preparations are presented in Table I.[3]

[1] The details of the modified method 10 were kindly provided by Dr. R. B. Pennell, of the Blood Characterization and Preservation Laboratory of the Protein Foundation, Inc., Jamaica Plain, Mass.

[2] Carrier-free I-131 was obtained from the Oak Ridge National Laboratory, Oak Ridge, Tenn.

[3] Note added in proof: Electrophoretic analysis of the I-131 anti-Rh_0 (D) preparations indicate that the fractions used in these studies all contained Fraction II + III instead of III alone.

TABLE I

Characterization of the I-131 anti-Rh_0(D) fractions

Property	Preparation A-15	Preparation A-18
Fraction	E III 1, 3	E III 0, 1, 3
% I-131 bound	10.9	13.2
M I/M globulin*	1.16	2.02
μc. I-131/μg. N†	0.94	0.75
% I-131 precipitable with trichloracetic acid	96.6	94.8
Titer before iodination	256	1,024
Titer after iodination	128	512
Nitrogen, μg. per ml.	1,080	2,270

* Calculated using a molecular weight of 160,000 for the total fraction iodinated.

† Activity at the time of iodination.

Determination of radioactivity. Measurement of I-131 gamma activity was carried out with a well-type scintillation detector using a thallium activated sodium iodide crystal (20) with four inches of lead shielding. The detector was coupled to a conventional scaler. The background counting rate for this detector, shielding and operating voltage (1,100 to 1,300) was 80 to 90 cpm. Detector efficiency was 7.03×10^5 cpm per μc. of I-131.[4] Sample and background counting rates were determined by using counting periods sufficient to maintain the total error below 5 per cent at the 0.05 level of significance (21). All samples had counting rates greater than three times background. Sample activity was maintained by volumetric dilution at a level which did not require correction for coincidence losses. Correction for radioactive decay was obviated by assaying a volumetrically diluted aliquot of the original I-131 isoantibody preparation with each sample throughout the course of the experiment. The radioactivity of all samples was converted to μg. nitrogen by use of the original I-131 reference sample. To check any variation in the efficiency of the counting equipment, the decay of the I-131 isoantibody standards throughout the course of the experiment was evaluated graphically.

Samples for radioactivity determination were prepared in 4 ml. capacity 15×45 mm. glass vials with Bakelite screw caps. Red cell suspensions were assayed by placing the Kahn reaction tubes directly in the well-counter.

Stroma preparation. Stroma preparations were derived from type O or A, Rh_0(D) positive red cells obtained from outdated units of whole blood. The stroma was prepared using the method described by Komninos and Rosenthal (22). The washed stroma was then lyophilized and maintained at $-20°$ C. until use. About 4 to 5 Gm. of stroma was obtained from 1 unit of whole blood with this technique

[4] Standardized against I-131 standards previously obtained from the National Bureau of Standards, Washington, D. C.

TABLE II

Properties of the I-131 anti-Rh_0(D) eluates

Preparation*	Elution procedure employed	T.C.A.† precipitable I-131	Titer	Nitrogen
				μg./ml.
A-15-1	Heat	87.2	1	0.399
A-15-2	Heat	89.5	4	0.604
A-15-3	Kidd method	80.9	Positive undiluted	0.201
A-18-1	Heat	84.7	Positive undiluted	0.076
A-18-2	Heat	80.4	Positive undiluted	0.316
A-18-3	Heat	90.3	1	0.332
A-18-4	Heat	94.9	1	0.380

* Alcohol fraction employed in obtaining eluate is indicated by the prefix of the preparation.
† Trichloracetic acid, 10 per cent.

Elution of I-131 anti-Rh_0(D) from Rh_0(D) stroma. From 250 to 500 mg. of lyophilized red cell stroma equivalent to 25 to 50 ml. of whole blood was sensitized by incubation with the I-131 isoantibody preparations. The sensitized stroma was washed three times with 0.15 M phosphate buffer, pH 7.5, and I-131 anti-Rh_0(D) was eluted from the washed sensitized stroma by the following modifications of Landsteiner and Miller's method (23). The eluting solution consisted of 3.0 ml. of 0.15 M phosphate buffer, pH 6.0, and either 0.5 ml. of Fraction III 1,3 (150 to 200 μg. of nitrogen) derived from type AB, Rh_0(D) positive plasma, or in some cases of 1.0 ml. type AB, Rh_0(D) positive plasma. When Fraction III 1,3 was used for the AB, Rh_0(D) positive carrier, 0.25 ml. of 30 per cent commercial bovine albumin was used in addition to increase the carrier protein. The sensitized stroma was eluted repeatedly for five times with the same volume of eluting medium at 56° C. (about twice the volume of the packed stroma). The first eluate was derived from a 5 minute incubation and the remaining four from a 15 minute period of incubation. The eluates were separated from the stroma by centrifugation at 56° C. by operating the centrifuge (Serofuge®, Clay Adams) in a 56° C. oven. In some experiments, the clarified eluates were passed through a column of Amberlite IR-45® in a 10 ml. syringe to remove unbound iodide-131. In the remaining experiments dialysis of the pooled eluates against phosphate buffer was substituted for this step. In a few studies the method described by Kidd (24) was used to elute the I-131 isoantibody from the sensitized stroma (Table II). The eluates after treatment with either the ion exchange resin or after dialysis were clarified by 10 minutes' centrifugation at maximum G 46,900 in a Spinco Model L Ultracentrifuge and merthiolate was added to the clarified eluates to yield a final concentration of 1 : 10,000.

The biological activity of each eluate was determined by reacting each eluate with both Rh_0(D) positive and negative red cells. The biologically active eluates were then pooled, dialyzed overnight against 0.15 M phosphate buffer, pH 6.5, and stored at 4° C. until used. The properties of the various eluates used in these studies are listed in Table II. Under these conditions of elution less than 2 per cent of the total I-131 added to the stroma was recovered in the eluates. A more detailed quantitative evaluation of the elution techniques used in these studies will be presented elsewhere.

Reaction of red cells with I-131 isoantibody preparations. Red cells were obtained from the donor population entering the Blood Bank. Venous blood was collected after phlebotomy was completed and heparin was used as the anti-coagulant. The cells were washed three times by centrifugation (International Clinical Centrifuge, Model CL) with a buffer wash solution consisting of 0.15 M phosphate buffer, pH 6.5, which contained by volume 10 per cent of a 0.16 M NaI, 0.016 M $NaHSO_3$ solution and 0.05 per cent of a commercial, 30 per cent bovine albumin. After the final wash the packed cells were resuspended in Alsever's solution equivalent to the original plasma volume and the red cells were stored at 4° C. Before use the cells were washed once with the buffer wash solution and resuspended in the same solution. All cells were used within five days after phlebotomy to avoid any changes induced in the red cell with these conditions of storage.

The cells were typed for the ABO and the Rh antigens using commercially obtained antisera. The techniques recommended by the manufacturer were employed and the results were controlled by using both positive and negative control cells.

Appropriate dilutions of red cells in the buffer wash solution were reacted in silicone-coated [5] Kahn serological tubes, 11 × 75 mm., with the I-131 isoantibody preparation. The red cell concentration used for most of these studies ranged from 2 to 10 volumes per cent. Cell concentration was determined by the microhematocrit method (25, 26). The capillary tubes were centrifuged (International Micro-hematocrit Centrifuge) for five minutes at maximum G 12,000. The red cells usually in a volume of 1 ml. were pipetted into the Kahn tubes containing the I-131 isoantibody with an Ostwald pipette. After addition of the red cells the tubes were stoppered with rubber stoppers, mixed and placed in a 37° C. water bath for one hour. The reaction tubes after incubation were centrifuged in 20 place bucketcups for three to five minutes at maximum G 1,650. The supernates were aspirated and saved for additional studies, and the cells washed twice with about 5 ml. of the buffer wash solution. The sensitized red cells were quantitatively transferred to a clean tube with Pasteur pipettes after the second wash, washed two additional times and then assayed for radioactivity. The determination of the red cell-bound I-131

[5] Dow-Corning 200, kindly provided by Dr. R. R. McGregor, Dow-Corning Corp. Midland, Mich.

TABLE III

Anti-$Rh_0(D)$ specificity of I-131 isoantibody preparation *

Probable cell genotype†	Total cpm bound to cells	% I-131 bound	Nitrogen bound
			$\mu g. \times 10^{-2}$
R^1R^2 (CDe/cDE)	583	5.79	2.20
R^2R^2 (cDE/cDE)	683	6.79	2.58
r'r (Cde/cde)	13	0.13	0.04
r''r (cdE/cde)	10	0.10	0.04
rr (cde/cde)	9	0.09	0.03

* Preparation A-18-1, 0.38 $\mu g.$ nitrogen and 10,065 cpm in 5 ml. with 0.3 ml. of an 8 per cent cell suspension. Cells incubated one hour at 37° C. Each value represents the average of two determinations.

† Cells obtained from a panel of Group O cells prepared by Knickerbocker, New York, N. Y.

was carried out by placing the Kahn tube directly into the well of the scintillation counter.

Calculations and assumptions employed. The interpretation of the data obtained in these studies depends on a number of assumptions. Two of these involve the calculations used to derive the molar ratio of iodine to specific isoantibody. One of these assumptions has been the use of 160,000 for the molecular weight of the anti-$Rh_0(D)$ and the other assumes that the iodine reacts equally and to the same extent with all the proteins in Fractions E-III 1,3 and E-III 0,1,3. Under these circumstances the iodine to nitrogen ratio of the total fraction would apply for all the proteins including the isoantibody. The finding that the iodine to nitrogen ratio of specific antibody is the same as that of the total gamma globulin fraction (18) offers support for this assumption. The stability of the iodine protein label *in vitro* has been confirmed by many studies (10). In view of this evidence the results obtained in these studies have been converted to nitrogen through the use of the iodine to nitrogen ratio of the total fraction that was iodinated. Although these studies do not provide direct experimental verification for these assumptions, valid and useful information can be obtained from these relative values (12).

A 1 ml. of a 10 per cent cell suspension was considered to be equivalent to 1.06×10^9 red cells. This value represents an approximation since red cells vary in volume from one individual to another. There is probably an even greater discrepancy when equating volumes per cent to number of red cells when the possible effects of storage on the red cell volume are considered.

Each sample of red cells was reacted with the I-131 isoantibody at least in duplicate and included a set of $Rh_0(D)$ negative cells as a control. The I-131 bound to the $Rh_0(D)$ positive cells was determined by subtracting the percentage of the total I-131 bound to the $Rh_0(D)$ negative cells from that found on the $Rh_0(D)$ positive cells. This corrected percentage of I-131 bound to the $Rh_0(D)$ positive cells was then converted to nitrogen through the use of the I-131 to nitrogen ratio of the total isoantibody-containing fraction, A-18 or A-15. The I-131 taken up by $Rh_0(D)$ negative cells was less than 3 per cent of that found on the $Rh_0(D)$ positive cells.

Justification for this correction is based on both the magnitude of the $Rh_0(D)$ negative cell value and the assumption that the I-131 bound nonspecifically to the $Rh_0(D)$ negative cells is also taken up by the $Rh_0(D)$ positive cells. The standard deviation of the overall determination of the amount of I-131 bound to a given red cell suspension was 2.6 to 1.5 per cent.

RESULTS

Iodine content and biological activity

There is no loss of biological activity with the introduction of one to two M iodine per M protein. The apparent decrease in titer after iodination (Table I) is due to dilution of the isoantibody which occurred during iodination.

I-131 binding by $Rh_0(D)$ negative red cells

$Rh_0(D)$ negative cells bind less than 2.5 per cent of the I-131 taken up by the $Rh_0(D)$ positive cells. The $Rh_0(D)$ specificity of the I-131 anti-

TABLE IV

Effect of antigen excess on quantity of I-131 bound to red cells *

Volume I-131 anti-$Rh_0(D)$	Total nitrogen added	Total cpm added to cells	Total cpm bound to $Rh_0(D)$ positive cells	Total cpm bound to $Rh_0(D)$ negative cells	Per cent I-131 bound			Nitrogen bound
					$Rh_0(D)$ positive cells	$Rh_0(D)$ negative cells	Corrected $Rh_0(D)$ positive cells	
ml.	$\mu g.$							$\mu g. \times 10^{-2}$
1.0	0.076	2,265	245	4	10.82	0.18	10.64	0.81
2.0	0.153	4,530	442	12	9.76	0.26	9.50	1.45
3.0	0.229	6,795	554	17	8.15	0.25	7.90	1.81
4.0	0.306	9,060	642	6	7.09	0.07	7.02	2.15
5.0	0.382	11,330	660	16	5.83	0.14	5.69	2.17

* Preparation A-18-3 reacted with 0.2 ml. of a 20 per cent cell suspension at 37° C. for one hour. Probable genotypes: O-R^1r (CDe/cde) or R^1R^0 (CDe/cDe) and O-rr (cde/cde).

TABLE V

Relationship of red cell concentration to antibody content *

Study	Total nitrogen added	Number of increments	Total time of incubation	Total cpm added to cells	Total cpm bound to $Rh_0(D)$ positive cells	Total cpm bound to $Rh_0(D)$ negative cells	Per cent I-131 bound			Nitrogen bound to cells
							$Rh_0(D)$ positive cells	$Rh_0(D)$ negative cells	Corrected $Rh_0(D)$ positive cells	
no.	*μg.*									*μg.* $\times 10^{-1}$
I	0.60	1	60	12,750	847	24	6.64	0.19	6.45	3.9
II	1.20	2	120	25,960	1,675	59	6.45	0.23	6.22	7.5
III	1.81	3	180	37,080	2,013	71	5.43	0.19	5.24	9.5
IV	1.81	1	60	37,170	1,736	27	4.67	0.07	4.60	8.3

* All determinations in triplicate. Preparation A–15–2 with 1 ml. of a 10 per cent red cell suspension was incubated at 37° C. for the time period indicated in the table. Probable genotypes are O–R^1r (CDe/cde) or R^1R^0 (CDe/cDe) and O–rr (cde/cde).

$Rh_0(D)$ isoantibody preparation is indicated in Table III. Less than 2.5 per cent of the I-131 taken up by $Rh_0(D)$ positive cells is bound by either rh′(C) or rh″(E) containing cells.

Relationship between isoantibody content and antigen sites

The effect of increasing the quantity of I-131 isoantibody on the total I-131 bound to $Rh_0(D)$ positive cells is shown in Table IV. As the volume of isoantibody reacted with a constant volume of cells is increased, the I-131 (nitrogen) bound progressively increases until a maximum value is reached. The addition of more isoantibody to the cells at this point is without effect on the quantity of I-131 (nitrogen) bound to the red cells. In this particular experiment the addition of more than 4 ml. of antibody resulted in antibody excess, so that saturation of all of the antigen sites had occurred. The reaction tubes with less than 4 ml. or 0.306 μg. of nitrogen are in the region of antigen excess where the number of antigen sites exceeds the available isoantibody.

The same relationship is also apparent from the data in Table V, Study I. This experiment in antigen excess, with only 0.6 μg. of isoantibody nitrogen, resulted in the binding of 0.039 μg. nitrogen. When three times the nitrogen was reacted with the same quantity of cells (Study IV) 0.083 μg. of nitrogen was bound. The addition of a second 0.6 μg. nitrogen increment of I-131 isoantibody to the cells of Study I resulted in the uptake of 0.075 μg. nitrogen. A third increment of I-131 isoantibody followed by a third incubation

TABLE VI

Specificity of the I-131 bound to red cells as determined by absorption studies

Source of supernate*	Total nitrogen added	Total cpm added to cells	Total cpm bound to $Rh_0(D)$ positive cells	Total cpm bound to $Rh_0(D)$ negative cells	Per cent I-131 bound			Nitrogen bound
					$Rh_0(D)$ positive cells	$Rh_0(D)$ negative cells	Corrected $Rh_0(D)$ positive cells	
	μg.							*μg.* $\times 10^{-2}$
$Rh_0(D)$ positive cells of Study I	0.47	8,931	70	15	0.78	0.17	0.61	0.3
$Rh_0(D)$ negative cells of Study I	0.50	9,335	584	17	6.26	0.18	6.08	3.0
$Rh_0(D)$ positive cells of Study IV	0.87	18,580	285†	12†	1.53	0.06	1.47	1.3
$Rh_0(D)$ negative cells of Study IV	0.94	19,490	1,010†	19†	5.18	0.10	5.08	4.8

* These supernates were obtained from the studies tabulated in Table V. The supernates reacted with 1 ml. of a 10 per cent red cell suspension for one hour at 37° C. Probable genotypes are: O–R^1r (CDe/cde) or R^1R^0 (CDe/cDe) and O–rr (cde/cde).

† Duplicate determinations, all others in triplicate.

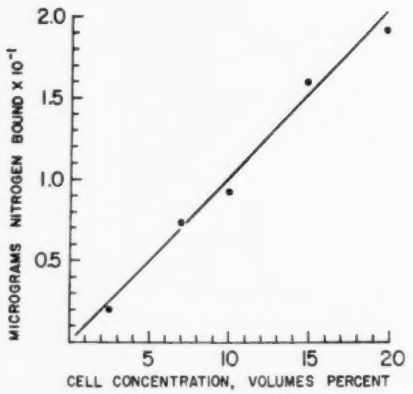

FIG. 1. EFFECT OF RED CELL CONCENTRATION ON THE QUANTITY OF I-131 BOUND TO THE RED CELL MASS

Each of the red cell concentrations indicated in the figure was suspended in 1 ml. buffer wash solution and incubated one hour at 37° C. with preparation A-15-1. Each value represents the average of two determinations. Probable genotypes: O-R¹r (CDe/cde) or R¹R° (CDe/cDe) and O-rr (cde/cde). Only the corrected $Rh_0(D)$ positive red cell values are plotted.

period resulted in a total of 0.095 μg. nitrogen bound to the red cells. These results indicate that the antigen sites on the red cell can be saturated by either reacting them with an excess of antibody or, if the reaction is in antigen excess, the antigen sites can be saturated by successive increments of antibody.

The reaction of the supernates obtained from Studies I and IV with $Rh_0(D)$ positive and negative red cells is shown in Table VI. The supernates recovered from the $Rh_0(D)$ negative red cells of Study I contained 0.03 μg. of specific nitrogen out of a total of 0.5 μg. or 6 per cent, which is comparable to the specific nitrogen content of the original preparation (6.5 per cent). The supernates obtained after reaction of the isoantibody with $Rh_0(D)$ positive red cells in Study I are almost free of I-131 isoantibody (0.003 μg. of nitrogen). The supernates from the $Rh_0(D)$ negative red cells of Study IV contain 0.048 μg. of specific nitrogen out of a total of 0.94 μg. of nitrogen, whereas the $Rh_0(D)$ positive red cell supernates from Study IV contain only 0.013 μg. of specific nitrogen. The antibody content of both supernates obtained from Study IV is inadequate to saturate all of the antigen sites present in 1 ml. of a 10 per cent red cell suspension. About 50 per cent of the antibody required was present in the $Rh_0(D)$ negative red cell supernates, and about 14 per cent in the $Rh_0(D)$ positive red cell supernates. The presence of antibody in the $Rh_0(D)$ positive red cell supernates of Study IV indicates that this study was carried out in antibody excess. The relationship between the I-131 bound to the red cell mass and the concentration of red cells plotted as volumes per cent is indicated in Figure 1. A direct proportionality exists between the amount of I-131 bound to the red cells and the concentration of red cells. The nitrogen bound to 1 ml. of a 10 per cent red cell suspension as estimated from these data is 0.092 μg.

Determination of the rate of reaction between anti-$Rh_0(D)$ and $Rh_0(D)$ positive red cells

The amount of I-131 bound to red cells as a function of time at 37° C. is shown in Figure 2. The curve obtained is an exponential growth curve that is consistent with a first order reaction, which follows the equation $A = A_0(1\text{-}e^{-kt})$, where A is the μg. nitrogen bound at time t, A_0 is the μg. nitrogen required to saturate all of the antigen sites and k is the reaction constant. A_0 for the cells used was 0.053 μg. nitrogen and k has a value of 5.78 per cent per minute or a half-time of 12 minutes.

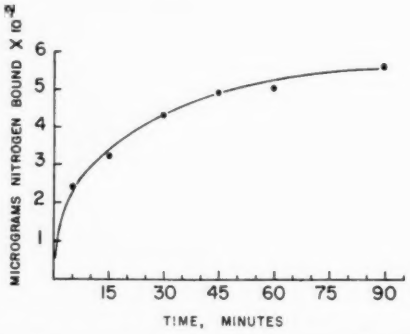

FIG. 2. EFFECT OF INCUBATION TIME ON THE QUANTITY OF I-131 BOUND TO THE RED CELLS

A 5 per cent red cell suspension in 1 ml. buffer wash solution was incubated at 37° C. with 2 ml. of preparation A-15-1, 0.80 μg. N, for the time indicated on the curve. Each value represents the average of three determinations. Probable genotypes: O-R¹r (CDe/cde) or r'R° (Cde/cDe) and O-rr (cde/cde). Only the corrected $Rh_0(D)$ positive red cell values are plotted.

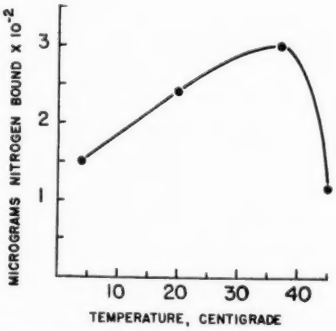

FIG. 3. EFFECT OF TEMPERATURE ON THE QUANTITY OF I-131 BOUND TO RED CELLS

Preparation A-15-3, 0.804 μg. nitrogen in 4 ml., was added to 1 ml. of a 5 per cent red cell suspension in buffer wash solution. Red cells were incubated one hour at the temperature indicated on the curve. Each value represents the average of two determinations. Probable genotypes: O-R^1r (CDe/cde) or R^1R° (CDe/cDe) and O-rr (cde/cde). Only corrected $Rh_0(D)$ positive red cell values are plotted.

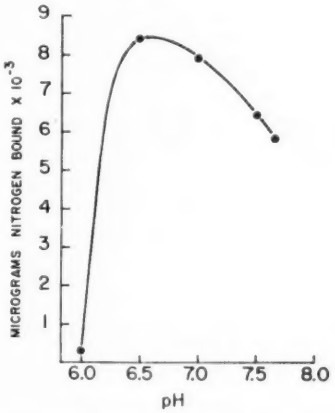

FIG. 4. EFFECT OF pH ON THE QUANTITY OF I-131 BOUND TO RED CELLS

Preparation A-18-1, 0.38 μg. of nitrogen in 5 ml., was added to 0.2 ml. of a 10 per cent red cell suspension. Both cells and the isoantibody preparation were made up in phosphate buffer with the pH indicated on the curve. Cells were incubated one hour at 37° C. Each value represents the average of two determinations. Probable genotypes: O-R°r (cDe/cde) or R°R° (cDe/cDe) and O-rr (cde/cde). Only corrected $Rh_0(D)$ positive red cell values are plotted.

Temperature and pH dependence of the reaction

The effect of temperature on the reaction between $Rh_0(D)$ positive red cells and I-131 anti-$Rh_0(D)$ is shown in Figure 3. The amount of I-131 bound to the red cells increases progressively from 4° C. to a maximum at 37° C. and then decreases markedly beyond 37° C.

The effect of pH on the quantity of I-131 bound to red cells is indicated in Figure 4. The maximum quantity of I-131 bound to the red cells occurs within a pH range of 6.5 to 7.0. On the acid side of this optimal pH region at pH 6.0 there is marked decrease in the I-131 taken up by the red cells. This effect seems to be associated with an increase in the I-131 bound to the $Rh_0(D)$ negative red cells. On the alkaline side of the optimal pH range there is a more gradual decrease in the quantity of I-131 bound to the red cells.

Inhibition studies

Specific blocking or inhibition of the I-131 bound to $Rh_0(D)$ positive red cells occurs when the cells

TABLE VII

Effect of pretreatment with unlabeled anti-$Rh_0(D)$ on the quantity of I-131 bound to red cells *

Pretreatment	Total cpm bound to $Rh_0(D)$ positive cells	Total cpm bound to $Rh_0(D)$ negative cells	Per cent I-131 bound			Nitrogen bound
			$Rh_0(D)$ positive cells	$Rh_0(D)$ negative cells	Corrected $Rh_0(D)$ positive cells	
						$\mu g. \times 10^{-3}$
Anti-$Rh_0(D)$ serum	21	7	0.10	0.03	0.07	1.1
O-$Rh_0(D)$ negative serum	411†	9†	1.98	0.04	1.94	30.6
PO_4 buffer control	374	8	1.80	0.04	1.76	27.7

* Cells consisted of 0.2 ml. of a 20 per cent cell suspension, incubated at 37° C. for one hour with sera indicated in column one, washed four times with buffer wash solution and reincubated with 5 ml. of I-131 anti-$Rh_0(D)$ preparation A-18-2, for one hour at 37° C. This volume of A-18-2 contained 1.575 μg. nitrogen and 20,740 counts per minute. Probable genotypes are: O-R^1R^1 (CDe/CDe) or R^1r' (CDe/Cde) and O-rr (cde/cde).

† Duplicate determinations; all others in triplicate.

TABLE VIII

Effect of heat on the ability of I-131 anti-$Rh_0(D)$ to bind to red cells *

Treatment of the I-131 anti-$Rh_0(D)$ preparation	Total μg. nitrogen added to cells	Total cpm added to cells	Total cpm bound to $Rh_0(D)$ positive cells	Total cpm bound to $Rh_0(D)$ negative cells	Per cent I-131 bound			Nitrogen bound
					$Rh_0(D)$ positive cells	$Rh_0(D)$ negative cells	Corrected $Rh_0(D)$ positive cells	
								μg. $\times 10^{-2}$
Unheated, control	1.32	10,450	516	2†	4.94	0.02	4.92	6.49
Heated 60° C. for 30 minutes	1.33	10,540	502	2†	4.76	0.02	4.74	6.31
Heated 70° C. for 30 minutes	1.27	10,020	303	2†	3.02	0.02	3.00	3.81

* Cells consisted of 1 ml. of a 5 per cent suspension incubated with 4 ml. of preparation A–18–3 for one hour at 37° C. Probable genotypes are: O-R^2r (cDE/cde) or R^2R^0 (cDE/cDe) and O-rr (cde/cde).

† Duplicate determinations; all others in triplicate.

are pretreated with a serum containing anti-$Rh_0(D)$ (Table VII). The red cells in this experiment were reacted at 37° C. for one hour with an unlabeled high-titered anti-$Rh_0(D)$ serum, a nonantibody containing O-$Rh_0(D)$ negative serum and phosphate buffer. The anti-$Rh_0(D)$ containing serum employed in this study was obtained from the same donor who provided the plasma that was used for preparing the I-131 anti-$Rh_0(D)$. Following the reaction of the cells with these three different preparations the cells were washed four times and reincubated with the I-131 anti-$Rh_0(D)$ preparation. The cells treated with the serum containing unlabeled anti-$Rh_0(D)$ took up 0.0011 μg. of nitrogen or less than 4 per cent of the nitrogen bound to the control cells.

Effect of heat on the biological activity of the I-131 anti-$Rh_0(D)$

The effect of heat on the biological activity of the I-131 anti-$Rh_0(D)$ was studied by heating two aliquots of the preparation, one at 60° C. and the other at 70° C. for 30 minutes. The heated preparations were then reacted with red cells (Table VIII). The biological activity of the preparation heated for 30 minutes at 60° C. was unaffected, whereas the preparation heated for 30 minutes at 70° C. retained only about 60 per cent of the activity found in the unheated preparation. For purposes of interpretation it should be noted that even the preparation labeled unheated has been subjected to 56° C. for about 70 minutes as a result of the elution technique that was used to ob-

TABLE IX

Effect of hemolysis on the I-131 bound to red cells *

Preparation assayed†	Total μg. nitrogen added to cells	Total cpm added to cells	Total cpm bound to $Rh_0(D)$ positive cells	Total cpm bound to $Rh_0(D)$ negative cells	Per cent I-131 bound			Nitrogen bound
					$Rh_0(D)$ positive cells	$Rh_0(D)$ negative cells	Corrected $Rh_0(D)$ positive cells	
								μg. $\times 10^{-2}$
I. Red cells	1.52	13,050	742	35	5.69	0.08	5.61	8.52
I. Stroma of above cells, centrifugation 15 min. at maximum 49,600 G	1.52	11,990‡	674‡	0‡	5.62	0.00	5.62	8.54
II. Red cells	3.04	21,890	1,060	13	4.84	0.06	4.78	14.5
II. Stroma of above cells, centrifugation 5 min. at maximum 1,650 G	3.04	20,330‡	585‡	10‡	2.88	0.05	2.83	8.60

* Values in table represent the average of duplicate determinations.

† Preparation A–18–4, 4 ml. for Study I and 8 ml. for Study II reacted with 1 ml. of a 10 per cent red cell suspension for one hour at 37° C. Study I was in antigen excess.

‡ Difference between these values and the red cell values is due to decay that occurred during the time that the stroma was prepared.

tain these preparations. Consequently, a truly unheated control was not used for this study.

Effect of hemolysis on red cell-bound I-131

Red cells were hemolyzed with distilled water after they had been sensitized with I-131 anti-Rh_0(D) (Table IX). In one experiment the red cell stroma was recovered by centrifugation in a Spinco Model L Ultracentrifuge for 15 minutes at maximum G 49,600 and in the other experiment the stroma was obtained by centrifugation for 5 minutes at maximum G 1,650 in a clinical centrifuge. With high-speed centrifugation the I-131 associated with the intact red cells was recovered completely in the centrifuged red cell stroma. With low-speed centrifugation only about 60 per cent of the red cell I-131 was found in the stroma. Failure to recover all the I-131 in the red cell stroma following low-speed centrifugation is probably due to incomplete recovery of the red cell stroma under these conditions of centrifugation.

DISCUSSION

The interpretation of the data obtained in these studies is based on the specificity of the I-131 bound to red cells (11–13). The effective exploitation of I-131 red cell isoantibodies depends on unequivocal evidence that the I-131 taken up by the red cells represents the specific isoantibody and conforms with the known behavior of these antibodies.

The first step in achieving this goal has been the recognition that red cell isoantibodies even in high-titered sera are present essentially in trace quantities, constituting probably less than 1 per cent of the plasma proteins (*27*). As a result efforts to concentrate the isoantibody by fractionation of the plasma are required. Even the concentration of the isoantibody by ethanol fractionation failed to yield a preparation which was effective following iodination. Only with further purification achieved by eluting the I-131 isoantibody from sensitized red cell stroma could preparations of sufficient biological activity be obtained.

A number of criteria have been employed to evaluate the nature and specificity of the I-131 preparations obtained with these techniques. The simplest procedure was to use Rh_0(D) negative cells with each experiment to evaluate the I-131 bound to red cells nonspecifically. Less than 3 per cent of the I-131 bound to the Rh_0(D) positive red cells was found on the Rh_0(D) negative red cells with the preparations used in these studies. In addition, both rh′(C) and rh″(E) red cells when reacted with the I-131 anti-Rh_0(D) bound less than 3 per cent of the I-131 found on the Rh_0(D) positive red cells. These findings indicate that the specificity of the I-131 isoantibody preparation is confined to the Rh_0(D) antigen.

The quantitative immunochemical nature of the reaction between the Rh_0(D) antigen and its isoantibody is evident from a number of the studies described.

The direct proportionality between the quantity of I-131 bound to the red cell mass and concentration of red cells (Figure 1) is consistent with the behavior of an antigen-antibody reaction. When an excess of red cells was used so that the available antigen sites exceeded the amount of antibody present, *i.e.*, antigen excess (Tables IV and V), the quantity of nitrogen bound to the red cells was less than that bound by the cells reacted at equivalence. Cells reacted under conditions of antigen excess and only partially saturated with antibody took up additional nitrogen when successively reacted with more antibody, until all the antigen sites were saturated. Absorption of the I-131 anti-Rh_0(D) with Rh_0(D) positive red cells quantitatively removed the I-131 capable of being bound to red cells.

Supernates obtained from reactions carried out at equivalence or antibody excess contained demonstrable quantities of I-131 that could be specifically bound to Rh_0(D) positive red cells (Table VI). In contrast absorption of the I-131 isoantibody preparation with Rh_0(D) negative red cells had no effect on the content of I-131 that was bound to Rh_0(D) positive red cells. These quantitative relationships between the Rh_0(D) red cell antigen and the I-131 isoantibody confirm inferences made previously (*28*) with unlabeled systems.

The specific nature of the I-131 bound to the Rh_0(D) positive red cells is supported by the inhibition or blocking studies (Table VII). In this experiment the red cell uptake of I-131 was com-

pletely blocked by treating the $Rh_0(D)$ positive red cells with a serum containing unlabeled anti-$Rh_0(D)$. These results provide direct evidence for the identity of the anti-$Rh_0(D)$ with the I-131 bound to the red cell.

Additional evidence for the specificity of the I-131 bound to red cells is provided by the reaction rate studies (Figure 2). The reaction between red cells and I-131 anti-$Rh_0(D)$ can be described as a first order reaction with a half-time of 12 minutes. The conventional incubation time of 60 minutes would result in saturation of 97 per cent of the available antigen sites when this constant is used in the calculation. It is apparent that the value obtained for the reaction constant using I-131 anti-$Rh_0(D)$ is consistent with the incubation time routinely employed for the detection of incomplete anti-$Rh_0(D)$.

The optimum temperature for the reaction between I-131 anti-$Rh_0(D)$ and red cells as determined by the maximum red cell uptake of I-131 occurred at 37° C. (Figure 3). This temperature is universally recognized as the optimum temperature for the detection of immune red cell isoantibodies (1–3). The marked drop in I-131 anti-$Rh_0(D)$ bound at 45° C. would also be expected in view of the widespread use of heat for the elution of isoantibodies from sensitized red cells (*22*, 23).

The pH dependence of the reaction (Figure 4) with the optimum between pH 6.5 to 7.0 confirms the studies of Carter (*29*) who found maximum Rh isoantibody titers at a pH of 7.0. The decrease on the acid side of the pH optimum probably is due in part to changes in the red cell which result in an increased susceptibility to hemolysis. If hemolysis occurs, loss of the stroma during conventional centrifugation could account for the decrease in the amount of I-131 bound to the red cells at pH 6.0.

Thermal stability is another property which has been used to characterize incomplete isoantibodies (1–3, 9). Heating the I-131 anti-$Rh_0(D)$ for 30 minutes at 60° C. (Table VIII) produced no decrease in the quantity of I-131 bound to $Rh_0(D)$ positive red cells. Even 30 minutes at a temperature of 70° C. did not completely abolish the ability of the I-131 anti-$Rh_0(D)$ to sensitize $Rh_0(D)$ positive red cells.

The classical interpretation of the red cell-isoantibody reaction postulates that the isoantibody is attached to the red cell stroma. Support for this view stems from the observation that antibodies can be eluted from stroma that has been obtained by hemolyzing antibody-coated red cells (*22–24*). The results of these studies with I-131 anti-$Rh_0(D)$ are consistent with this interpretation of the red cell-isoantibody reaction. Complete recovery of all the I-131 anti-$Rh_0(D)$ bound to red cells was obtained in the red cell stroma after hemolysis (Table IX). Failure to obtain all of the I-131 in the stroma following low-speed centrifugation is probably due to loss of some of the stroma with these conditions of centrifugation.

An estimate of the number of antigen sites on each red cell can be made using these data if the assumptions discussed previously are granted. At the present time no value has been assigned to the molecular weight of the $Rh_0(D)$ isoantibody. The value 1.6×10^5 for the molecular weight (30) has therefore been used in these calculations. The other assumption is that the iodine to nitrogen ratio of the specific antibody is the same as that of the total alcohol fraction. The agreement in the quantity of nitrogen bound to $Rh_0(D)$ positive red cells between two different fractions, A-18 and A-15, that differed both in nitrogen content and in protein content (A-18 contained the lipoproteins) suggests that the iodine content of all the proteins in the fraction is proportional to the protein nitrogen.

An additional approximation which is necessary to calculate the number of antigen sites on a red cell assumes that the red cell volume is directly proportional to the red cell number. Obviously this approximation will not hold up in comparing red cells of one individual to those of another. This assumption is probably even less reliable when applied to red cells that have been stored *in vitro* in view of the tendency of red cells to undergo changes in volume under these conditions.

The maximum number of $Rh_0(D)$ sites on each red cell obtained by using these assumptions is 2,000 to 3,000. The calculations involve converting the nitrogen bound to number of molecules by means of the molecular weight and Avogadro's number. The number of molecules bound to the cell mass is then divided by the estimated number of cells, 1.064×10^9 in 1 ml. of a 10 per cent cell suspension. This value is about one-half of that

reported previously (11). The discrepancy is probably related to the fact that these authors iodinated whole serum rather than plasma protein fractions.

The data, interestingly enough, also suggest that the Rh_0(D) positive red cells can be segregated into two groups in terms of the quantity of nitrogen bound. About 60 per cent of the cells used in these studies take up about 0.10 μg. of nitrogen and the remaining 40 per cent of the cells, about 0.05 μg. of nitrogen. The difference between these two populations of cells may be related to homozygosity for Rh_0(D) in the cells that bound 0.10 μg. of nitrogen and to heterozygosity for Rh_0(D) in the cells that bound in the range of 0.05 μg. of nitrogen. More definitive studies will be required to firmly establish the one to one correspondence between genetic constitution and the number of antigen sites on the red cell, in view of the assumptions that were used in these calculations.

The results obtained in these studies with I-131 anti-Rh_0(D) confirm and provide direct evidence for the classical interpretation of the reaction between red cells and isoantibodies. The I-131 labeled anti-Rh_0(D) has enabled direct observation and quantitation of the physical attachment of the antibody to the red cell. These observations lend support to the concept that the "sensitization" of the red cell is independent of agglutination. The nature of the attachment of the antibody to the red cell is consistent with the concept that the red cell has discrete antigen sites on the stroma and that the antibody is firmly and quantitatively bound to these sites. Extension of these techniques to other red cell antigens should provide a better understanding of many problems in the field of hematology, blood banking and human genetics.

SUMMARY

1. Trace labeled I-131 anti-Rh_0(D) was prepared by iodinating isoantibody-containing fractions obtained by alcohol fractionation of high-titered anti-Rh_0(D) plasma. An I-131 anti-Rh_0(D) was prepared by eluting the anti-Rh_0(D) from red cell stroma sensitized with the I-131 alcohol fractions (E-III 0,1,3, and E-III 1,3).

2. The evidence for the specificity of the I-131 bound to red cells following the use of these preparations consists of the following observations:

a. Similarly treated Rh_0(D) negative red cells bind less than 3 per cent of the I-131 taken up by the Rh_0(D) positive red cells.

b. Selective removal or absorption of the I-131 anti-Rh_0(D) by Rh_0(D) positive red cells and failure of Rh_0(D) negative red cells to absorb or remove the I-131 anti-Rh_0(D) activity has been demonstrated.

c. A direct proportionality exists between the amount of I-131 bound to the Rh_0(D) positive red cells and the concentration of red cells.

d. Complete inhibition of the binding of I-131 occurs when the red cells have been pretreated (sensitized) with unlabeled anti-Rh_0(D) serum.

e. The reaction between I-131 anti-Rh_0(D) and Rh_0(D) positive red cells has been characterized as a first order reaction with a rate constant of 5.78 per cent per minute.

f. The temperature and pH optima as well as the thermal stability of the I-131 anti-Rh_0(D) preparation are similar to those found with the native anti-Rh_0(D).

g. The I-131 bound to red cells has been quantitatively recovered in the stroma after hemolysis of I-131 sensitized red cells.

3. An estimate made from these data indicates that there are 2,000 to 3,000 Rh_0(D) antigen sites on each red cell.

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EFFECTS OF EXOGENOUS ADRENOCORTICOTROPIC HORMONE (ACTH) UPON PITUITARY ACTH CONCENTRATION AFTER PROLONGED CORTISONE TREATMENT AND STRESS *

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Symptoms and metabolic abnormalities closely resembling those of hypoadrenalism have been reported following cessation of prolonged treatment with adrenal steroids (1–5). Sudden death following surgery has been reported in association with this syndrome and has been ascribed to failure of the hypothalamic-pituitary-adrenal system to respond to stress (6–9). Since prolonged administration of corticosteroids produces adrenal atrophy in both man and laboratory animals (7, 10, 11), this failure has been attributed to adrenal insufficiency. Exogenous corticotropin (ACTH) will repair or maintain normal adrenal weight in the cortisone-treated animal (12). Consequently the addition of ACTH in various dosage schedules has been advocated for the purpose of maintaining or restoring normal adrenal responsiveness to stress (5, 13–16). The efficacy of such combined therapy is not firmly established.

In addition to producing adrenal atrophy, chronic treatment with steroids results in decreased pituitary ACTH content (17, 18). The failure of steroid-treated subjects to respond to stress may therefore reflect pituitary ACTH insufficiency rather than, or in addition to, adrenal insufficiency. ACTH administration, on the other hand, has been shown to increase pituitary ACTH content in the intact rat, despite stimulation of secretion of endogenous steroids (18). For this reason ACTH therapy has been studied in the cortisone-treated animal to determine whether such adjunctive therapy is capable of modifying the pituitary ACTH depletion which follows steroid administration, and whether it can enhance the pituitary response to stress.

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‡ Commonwealth Fund Fellow in Medicine.

METHODS

All experiments were performed in male rats of the Sherman strain (100 to 140 Gm.), kept in a constant temperature room for at least one week prior to use. The diet consisted of Purina Laboratory Chow and water *ad libitum*. Three treatments were employed as follows: Physiological saline (0.1 ml.) was injected subcutaneously once daily; 4 U.S.P. units of ACTH gel (0.1 ml.) was injected subcutaneously once daily; 2.5 or 5 mg. of cortisone acetate (either 0.1 or 0.2 ml.) was injected subcutaneously once daily. When adrenalectomized rats were used they were injected immediately after operation and were given 1 per cent saline in the drinking water.

Four experiments were performed as follows:

Experiment 1. Intact rats were injected with 5 mg. of cortisone for 7 days. Groups of four animals each were sacrificed at 1, 2, 4, 7 and 10 days after the last injection. The pituitary glands of each group were pooled and extracted for ACTH. Intact rats injected with saline for 7 days and sacrificed 24 hours after the last injection served as a control group.

Experiment 2. Adrenalectomized rats were injected with either 5 mg. of cortisone or 4 U.S.P. units of ACTH plus 5 mg. of cortisone for 7 days. A control group received saline. The pituitary glands were extracted individually for ACTH 24 hours after the last injection.

Experiment 3. Intact rats were divided into five groups and treated according to the following 10 day schedule:

Group	Treatment 1 (seven days)	Treatment 2 (three days)
1 (control)	Uninjected	Uninjected
2	Cortisone, 2.5 mg.	Saline
3	Cortisone, 2.5 mg. plus ACTH, 4 U	Saline
4	Cortisone, 2.5 mg.	ACTH, 4 U
5	Cortisone, 2.5 mg. plus ACTH, 4 U	ACTH, 4 U

Twenty-four hours after completion of the injection schedule the animals in each group were subdivided into *unstressed* and *stressed* subgroups. Four hours after a scalding stress (19), both the unstressed and stressed subgroups were sacrificed simultaneously. This interval was chosen on the basis of previous experiments in which normal rats were scalded and sacrificed 1, 2, 4, 8 and

24 hours after stress; the maximal depletion of pituitary ACTH was observed to occur at 4 hours. The pituitary glands of each subgroup were pooled and extracted for ACTH.

Experiment 4. Intact rats were divided into two groups and treated according to the following 10 day schedule:

Group	Treatment 1 (seven days)	Treatment 2 (three days)
1 (control)	Cortisone, 2.5 mg.	Saline
2	Cortisone, 2.5 mg. plus ACTH, 4 U	Saline

Twenty-four hours after the last injection the animals in each group were subdivided into *unstressed* and *stressed* subgroups. Four hours after the scalding stress, both the unstressed and stressed subgroups were sacrificed simultaneously. The pituitary glands of each subgroup were pooled and extracted for ACTH.

The pituitary glands of the experimental animals were extracted for ACTH according to the method of Birmingham and co-workers (20). These extracts were subsequently assayed for ACTH by incubation with rat adrenal slices *in vitro* by a modification of the method of Saffran and Schally (21) previously described (18). The pituitary extracts of the appropriate control animals were each assayed against a standard ACTH preparation. The absolute potencies of these control extracts are expressed as concentration of ACTH per mg. anterior pituitary tissue, and are included in Tables I, II and III. The pituitary extracts of the appropriate control animals then were employed as "house" standards in each experiment and assigned arbitrary potencies of 100 per cent. The pituitary ACTH concentrations in the various experimental groups are expressed as per cents of their respective controls. These percentages represent an arithmetic conversion of the mean log potency ratios obtained and are presented in this form solely for purposes of clarity. The absolute potencies of the various experimental groups can be derived by simple multiplication of these per cents by the absolute potencies of the appropriate control extracts. Comparisons of differences in ACTH potency were determined from the mean log potency ratios and their respective log standard errors ($\overline{M} \pm s_{\overline{M}}$) and not upon the arithmetic percentages (antilog $\overline{M} \pm 2.303\, s_{\overline{M}}$ antilog $\overline{M}$) presented in the tables. The data are calculated in the form of concentration of ACTH per mg. anterior pituitary tissue. Total ACTH content of the individual pituitary glands or pools was also calculated; however, in no instance do the values for ACTH content deviate significantly from those presented for concentration. Individual pituitary glands in Experiment 2 were assayed once each and the pituitary pools in Experiments 1, 3 and 4 were assayed two to four times each. Statistical evaluations were calculated according to the methods of Bliss (22) and Snedecor (23).

TABLE I

Changes in pituitary adrenocorticotropic hormone (ACTH) concentration in intact rats following termination of chronic cortisone administration

Days after last cortisone injection	Number of rats	Pituitary ACTH as % control level*	Combined adrenal weight†
			mg.
Control	9	100‡	
1	4	41 ± 9§	16 ± 3
2	4	32 ± 6§	16 ± 1
4	4	28 ± 5§	16 ± 1
7	4	47 ± 8§	16 ± 4
10	4	58 ± 11§	16 ± 3

* Antilog $\overline{M} \pm 2.303\, S_{\overline{m}}$ (antilog $\overline{M}$).
† Mean ± standard error of the mean.
‡ Absolute potency, 41 mU ACTH per mg.
§ $p \leq 0.01$.

TABLE II

Effects of concurrent ACTH administration upon pituitary ACTH depletion caused by cortisone in adrenalectomized rats

Group	Number of rats	Pituitary ACTH as % control level*
Saline (control)	10	100†
Cortisone	7	62 ± 7‡
Cortisone plus ACTH	8	74 ± 5‡

* Antilog $\overline{M} \pm 2.303\, S_{\overline{m}}$ (antilog $\overline{M}$).
† Absolute potency, 50 mU ACTH per mg.
‡ $p \leq 0.01$.

RESULTS AND DISCUSSION

Although pituitary ACTH depletion is known to occur after prolonged steroid administration (17, 18), no data are available concerning the duration of this depletion following cessation of the steroid therapy. A significant fall in pituitary ACTH concentration to 41 per cent of control occurs in cortisone-treated rats 24 hours after the last injection when compared to saline-injected controls (Table I). However, the maximal depletion to 28 per cent of control is not observed until 4 days following treatment. This possibly reflects a continuing effect of cortisone due to delayed release of the steroid from the sites of injection. Thereafter, pituitary ACTH concentration increases gradually with a significant increment above the 4 day value noted 10 days after treatment. However, the latter level is still significantly lower than the control value. Despite this repletion of pituitary ACTH, repair of adrenal atrophy has not

TABLE III

Effects of exogenous ACTH upon pituitary ACTH concentration after steroid withdrawal and stress in intact rats

Group			Before stress		After stress		
No.	Treatment 1	Treatment 2	No. of rats	Pituitary ACTH as % control level*,†	No. of rats	Pituitary ACTH as % control level*,‡	Combined adrenal weight§,‖
							mg.
Experiment 3							
1	Uninjected	Uninjected	8	100 (control)¶	3	34 ± 10**	25 ± 1 (14)
2	Cortisone	Saline	3	39 ± 8**	3	20 ± 8**	14 ± 1 (9)
3	Cortisone plus ACTH	Saline	3	53 ± 7**	3	18 ± 3**	17 ± 1 (9)
4	Cortisone	ACTH	3	33 ± 8**	4	22 ± 3	18 ± 1 (10)
5	Cortisone plus ACTH	ACTH	3	29 ± 4**	4	26 ± 4	21 ± 1 (10)
Experiment 4							
	Cortisone	Saline	4	100 (control)	5	40 ± 6**	
	Cortisone plus ACTH	Saline	4	81 ± 12	6	43 ± 7**	

* Antilog $\overline{M} \pm 2.303\ S_{\overline{m}}$ (antilog $\overline{M}$).

† Calculation of significance of effects of treatment on pituitary ACTH before stress based on comparison with unstressed, uninjected control group.

‡ Calculation of significance of pituitary ACTH depletion after stress based on comparisons with respective pre-stress groups.

§ Mean ± standard error.

‖ Number of animals studied indicated in parentheses.

¶ Absolute potency, 63 mU ACTH per mg.

** $p \leq 0.01$.

begun by the tenth day (Figure 1). These data are compatible with the hypothesis that ACTH release is negligible after cessation of cortisone treatment in the unstressed rat and that the ACTH synthesized during this period is stored rather than secreted. Prolonged adrenal atrophy after cortisone therapy has been observed previously in the rat (11) and also in man (7); in the latter report, adrenal atrophy (associated with sudden death following stress) was seen four and one-half months after completion of steroid treatment.

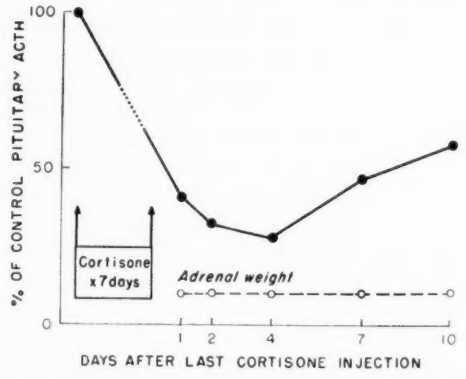

FIG. 1. CHANGES IN PITUITARY ACTH CONCENTRATION AND ADRENAL WEIGHT FOLLOWING CORTISONE ADMINISTRATION FOR SEVEN DAYS

Graphic representation of data presented in Table I. Note persistent adrenal atrophy despite evidence of rising pituitary ACTH concentration 10 days following last cortisone injection.

Since prolonged cortisone administration results in marked pituitary ACTH depletion, Experiments 2, 3 and 4 were designed to determine whether ACTH treatment can alter the pituitary ACTH content of cortisone-treated rats. A significant fall to 62 per cent of control occurs in adrenalectomized rats treated daily with 5 mg. cortisone alone compared to saline-injected controls (Table II). Concurrent administration of ACTH results in a significant fall to 74 per cent of the control level. The apparent difference of 12 per cent, although not significant, suggests that combined therapy may partially overcome the depletion of pituitary ACTH concentration induced by very large doses of cortisone.

Various schedules of combined ACTH and steroid therapy have been advocated for clinical use, *e.g.*, administration of ACTH concurrently with steroids, ACTH following cessation of steroid treatment or ACTH both during and after steroid therapy. Consequently, these injection

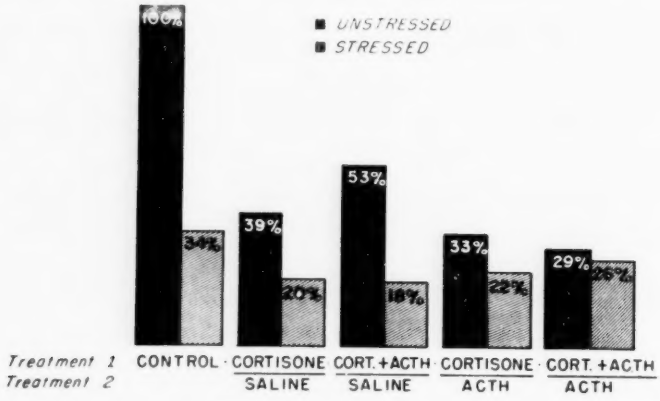

FIG. 2. EFFECTS OF ACTH ADMINISTRATION UPON PITUITARY ACTH CONCENTRATION IN CORTISONE-TREATED RATS BEFORE AND AFTER STRESS

Graphic representation of data presented in Table III. Pituitary ACTH concentrations are expressed as percentages of the control (uninjected) unstressed subgroup.

schedules were followed in Experiment 3 in addition to a group receiving cortisone without ACTH and an uninjected control group (Table III).

Animals treated with 2.5 mg. of cortisone without suplementary ACTH showed a depletion in pituitary ACTH concentration to 39 per cent of the control level four days after steroid withdrawal. This decrease is consistent with that seen in Experiment 1, in which twice the dose of cortisone caused a fall in pituitary ACTH to 28 per cent of the control level at a comparable interval following cessation of steroid administration.

No significant modification of this pituitary ACTH depletion is observed in any of the groups receiving exogenous ACTH in addition to steroids. As in Experiment 2, however, the animals injected with ACTH and cortisone concurrently (Group 3) demonstrate a pituitary ACTH concentration suggestively, but not significantly, greater than that obtained in animals given cortisone alone.

The hypothesis that exogenous ACTH is capable of improving the pituitary response of rats stressed after withdrawal of cortisone was tested by determining the effects of a scalding stress on pituitary ACTH concentration (Table III, Figure 2). Pituitary ACTH in uninjected rats after scalding is 34 per cent of the control level, representing a fall of 66 per cent. On the other hand, the rats pretreated with cortisone (Group 2) show a poststress pituitary ACTH level of 20 per cent, in contrast to the pre-stress level of 39 per cent. This difference of 19 per cent, although statistically significant, is less than one-third that obtained in control animals. These findings suggest that ACTH release in response to stress is markedly reduced in rats during the period of steroid withdrawal.

It is assumed, in the above interpretation of the data, that the markedly smaller difference between pre- and poststress pituitary ACTH concentration in cortisone-treated rats primarily reflects diminished ACTH release. It is recognized that pituitary ACTH levels at any moment represent the algebraic sum of the synthesis and the release of ACTH. Negative ACTH balance, *i.e.*, a decrease in pituitary ACTH level, may reflect reduced synthesis, accelerated release, or both conditions occurring concurrently. Positive ACTH balance, *i.e.*, an increase in pituitary ACTH level, may represent accelerated synthesis, reduced or blocked release, or a combination of the two. When no change in ACTH balance is observed after an experimental procedure, no conclusion can be drawn as to the rates of synthesis and release, other than that the two processes are in equilibrium. In Experiment 3 (Table III, Fig-

ure 2), the fall in pituitary ACTH following stress is over three times as great in the control animals (Group 1) as in the cortisone-treated rats (Group 2). If one proposes that equal quantities of ACTH were released by the two groups, then a rate of synthesis three times normal must be assumed for the group pretreated with cortisone. This last assumption appears untenable, in view of the data presented in Experiment 1 in which treatment with cortisone resulted in a severe depression of pituitary ACTH synthesis, as manifested by a striking and persistent decrease in pituitary ACTH content and adrenal weight. Therefore, a more appropriate explanation appears to be that the threefold difference in negative ACTH balance between Groups 1 and 2 after stress represents primarily a difference in the amounts of ACTH released by the two groups. The only assumption underlying this interpretation is that ACTH synthesis in the two groups is equivalent; the data of Experiment 1 indicate that this assumption is quite conservative. These considerations also apply to the discussion of the data which follow.

The rats pretreated with ACTH concurrently with cortisone (Group 3) also demonstrate a significant further reduction in pituitary ACTH as a result of stress. However, the change observed does not differ significantly from that obtained in rats given cortisone alone (Group 2), and is similarly much reduced in comparison to that observed in control animals. In contrast to the foregoing groups, rats injected with ACTH consecutively (Group 4) or concurrently plus consecutively (Group 5) do not exhibit significant falls in pituitary ACTH after the scalding stress. These data would suggest that ACTH therapy, when continued to the time of stress as in these last two groups, actually inhibits the release of ACTH from the pituitary gland. The findings are consistent with previous experiments (19) which demonstrated that administration of exogenous ACTH prevented release of endogenous ACTH in response to stress, via an extra-adrenal mechanism.

Definite adrenal atrophy persists following withdrawal of cortisone (Table III, Figure 3). All of the groups receiving ACTH in addition to the cortisone show significant increments in adrenal weights, as would be expected. The most effective maintenance of adrenal weight is seen in those animals receiving ACTH both during and following cortisone treatment (Group 5). When the mean adrenal weights are adjusted for variations in body weight by analysis of covariance, this group actually shows hypertrophy of the adrenals when compared to controls.[1] However, this group manifested the most significant inhibition of pituitary ACTH release in response to stress (*vide supra*).

Since the data suggest that concurrent administration of ACTH to the cortisone-treated animal increases pituitary ACTH concentration following steroid withdrawal, a more precise experimental design was employed (Experiment 4), which involved a direct comparison of pituitary ACTH in the group receiving combined therapy to the group receiving cortisone alone, the latter

[1] Adjustment of mean adrenal weights for variations in body weight does not otherwise alter the significant differences depicted in Figure 3.

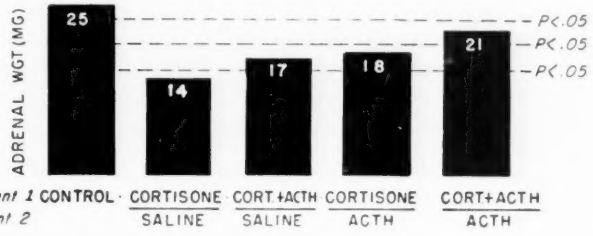

FIG. 3. EFFECTS OF ACTH ADMINISTRATION UPON ADRENAL WEIGHT IN CORTISONE-TREATED RATS

Means of the combined adrenal weights in the control and treatment groups of Experiment 3. Standard errors of these means are presented in Table III. Dotted lines indicate significant differences (at the 5 per cent level of confidence) among the various mean adrenal weights.

serving as control (Table III). No significant difference between the pituitary ACTH concentrations of the two groups is seen, however, and the depletions in pituitary ACTH following stress are significant and similar in both groups when each is compared to its unstressed control.

It would appear then that in the rat receiving prolonged steroid therapy the administration of ACTH does not prevent or ameliorate the depletion of pituitary ACTH which results after cortisone. Nor is the diminished amount of ACTH released in response to severe stress increased by such therapy. The rat receiving ACTH up to 24 hours before stress shows the blockading effect of exogenous ACTH upon pituitary ACTH release in response to stress (19). However, administration of ACTH does prevent or repair adrenal atrophy in steroid-treated animals. In view of the inhibition of ACTH synthesis and release which has been demonstrated in animals receiving either cortisone alone, or combined treatment, the significance of this effect of ACTH is uncertain. It is possible that, with the ACTH-induced preservation of adrenal mass, the adrenal response to stress may be normal during the period of steroid withdrawal, despite the severe reduction in ACTH release. In the absence of data concerning adrenal steroid production under these conditions, no conclusions may be drawn concerning the adrenal response to stress. It should be emphasized that measurement of pituitary, and not adrenal, responsiveness to stress is the object of the present experiments.

The failure of exogenous ACTH to raise pituitary ACTH concentration in the steroid-treated rat in the above experiments is in striking contrast to the rise in pituitary ACTH seen following ACTH treatment in the normal or adrenalectomized rat (18, 19). The fall in pituitary ACTH after cortisone treatment is thought to represent inhibition of ACTH synthesis, and perhaps also inactivation of stored pituitary ACTH. Exogenous ACTH apparently is unable to reverse the steroid-induced inhibition of ACTH synthesis and depletion of ACTH stores.

Uncautious extrapolations from these studies to the clinical problems associated with steroid administration and withdrawal are not warranted. It is important to note that the dosages of hormones used in these experiments, calculated on the basis of body weight, represent amounts far in excess of those usually employed in the treatment of human disease. Moreover, important species differences may well exist between man and the rat insofar as pituitary-adrenal physiology is concerned.

However, clinical observations by Kyle, Meyer and Canary (24) in patients with Cushing's syndrome due to adrenal tumors correlate quite closely with the present animal studies. Adrenal insufficiency is frequently encountered following resection of such neoplasms. This has been attributed to the associated atrophy of the contralateral adrenal gland (25). Kyle's two patients, however, manifested persistent adrenal hypofunction for one to two years postoperatively after cessation of repeated courses of ACTH. Although ACTH was capable of stimulating the adrenals to produce normal levels of steroids, discontinuation of ACTH therapy was quickly followed by relapse into the hypoadrenal state. It was concluded, therefore, that the basic lesion in these patients was located in the pituitary gland rather than in the adrenals, since therapy directed at restoring adrenal weight was incapable in itself of returning adrenal function to normal.

On the other hand, Bondy and co-workers (26, 27) considered hypothalamic-pituitary-adrenal function to be normal in patients who had received prolonged ACTH and steroid therapy, despite the presence of "steroid withdrawal" symptoms. A conclusion of normal pituitary responsiveness was inferred from a rise in plasma corticoid level following the stress of hypoglycemia. The discrepancy between this conclusion and that obtained by direct measurements in rats in the present study perhaps may be explained by differences in the species and treatments employed. Final conclusions must await the development of techniques for the direct evaluation of pituitary ACTH reserve in man.

SUMMARY

1. Pituitary adrenocorticotropic hormone (ACTH) concentration is significantly depleted following chronic administration of cortisone in the rat. Persistence of adrenal atrophy despite partial repletion of pituitary ACTH levels 10 days after cessation of treatment suggests that ACTH release during this period is negligible and that

newly-synthesized ACTH is stored rather than secreted.

2. The steroid-induced depletion of pituitary ACTH concentration is neither prevented nor ameliorated by the addition of ACTH in a variety of dosage schedules. Adjunctive ACTH administration does not modify the striking reduction in ACTH release after stress which occurs in the cortisone-treated animal.

3. Adrenal atrophy following steroid treatment can be prevented or repaired by the administration of ACTH. The significance of this action of ACTH is uncertain, in view of the derangements in pituitary ACTH synthesis and release which are observed to exist in animals receiving either combined therapy or cortisone alone.

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ON THE RATE OF HYDROCORTISONE CLEARANCE FROM PLASMA IN PREGNANT WOMEN AND IN PATIENTS WITH LAENNEC'S CIRRHOSIS *

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The question of whether or not there is an increase in adrenal cortical secretion during pregnancy remains unsettled. Morphologic studies of the adrenal during gestation have revealed enlargement of the gland in some species (1) and no enlargement in others (2). In the human female, several groups of investigators have observed a progressive rise in the plasma 17-hydroxycorticosteroid (17-OH-corticosteroid) level during pregnancy (3–8). Paper chromatographic studies (9), confirmed in this laboratory (10), have indicated that the elevated plasma 17-OH-corticosteroid level is due to an increase in circulating hydrocortisone, and not to a nonspecific chromogen. Measurement of urinary 17-OH-corticosteroids has given equivocal results. Jayle, Desgrez, Serpicelli and Rozeg (11), Devis (12), Mills (13), and Appleby and Norymberski (14) found that the rise in urinary 17-OH-corticosteroids and 17-ketogenic steroids during pregnancy was of only moderate degree. Contradictory results have been obtained from studies of urinary 11-oxygenated 17-ketosteroids, the principal C19 metabolites of hydrocortisone (15). Dobriner, Lieberman, Rhoads and Taylor (16) and Davis and Plotz (17) reported that urinary 11-oxygenated 17-ketosteroids did not increase with advancing gestation, while Huis in't Veld (18) and Birke, Gemzell, Plantin and Robbe (19) claimed that these steroids were present in increased quantity in the third trimester.

In an attempt to reconcile the apparent discrepancy between the finding of elevated plasma and of relatively normal urinary 17-OH-corticosteroid levels in pregnancy, Migeon, Bertrand and Wall investigated the rate of disappearance of injected 4-C^{14}-hydrocortisone from plasma of pregnant women near term and found it to be delayed (20). These workers also observed a reduced rate of appearance of plasma and urine 17-OH-corticosteroid conjugates (glucuronides) after the administration of labeled hydrocortisone. These data suggested that the co-existence of high plasma (unconjugated) 17-OH-corticosteroid values and normal urine (unconjugated plus conjugated) 17-OH-corticosteroid values might be partly explained by the presence in both plasma and urine of a greater proportion of free hydrocortisone and a smaller proportion of conjugated hydrocortisone metabolites than normal (20). Devis (12) and Gray (21) have reported increased amounts of free 17-OH-corticosteroid in the urine of pregnant women.

The present report describes studies upon the rate of hydrocortisone clearance from plasma in late pregnancy and in Laennec's cirrhosis, a condition in which hydrocortisone disposal rate is reportedly delayed (22–24). The data suggest that a delay in hydrocortisone clearance may not completely account for the elevated plasma 17-OH-corticosteroid levels observed in the pregnant state.

MATERIALS AND METHODS

Patients. Subjects were pregnant women in the third trimester of pregnancy who were not acutely ill and who showed no evidence of rheumatic heart disease, diabetes mellitus or pre-eclampsia. The women were hospitalized for premature rupture of the membranes or in anticipation of elective Caesarian section. Patients with Laennec's cirrhosis were six men and one woman with the typical clinical and laboratory features of parenchy-

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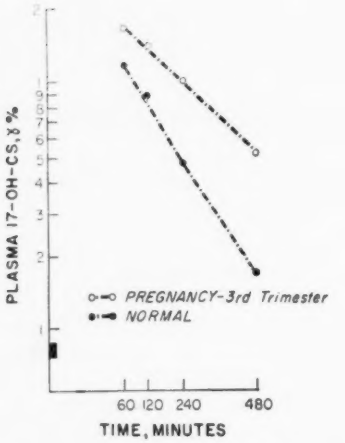

FIG. 1. SEMILOGARITHMIC PLOT OF THE RATE OF FALL OF PLASMA 17-OH-CORTICOSTEROID LEVELS AFTER INTRAVENOUS INJECTION OF 100 MG. HYDROCORTISONE

Average per cent decrease in level (per hour) for pregnant women (15 per cent) is significantly less than that for normal subjects (27 per cent) at the 1 per cent level.

m.[1] liver disease who exhibited minimal jaundice and ascites at the time of study. Normal subjects were female hospital patients of child-bearing age who had no endocrine disease and who were not acutely ill, or presumably healthy female laboratory workers.

Analytic methods. Free plasma 17-OH-corticosteroid levels were determined by the Silber-Porter method (25) as modified in this laboratory (26). Plasma 17-OH-corticosteroid glucuronides (presumed to be largely the glucuronides of 3 alpha, 17 alpha, 21-tri-hydroxypregnane-11,20-dione or tetrahydrocortisone, and of 3 alpha, 11 beta, 17 alpha, 21-tetrahydroxypregnan-20-one or tetrahydrocortisol) were measured as Porter-Silber chromogen following beta-glucuronidase hydrolysis for 48 hours according to a modified Bongiovanni-Eberlein procedure (22). Free hydrocortisone was first extracted exhaustively from plasma samples with dichloromethane (three times with fivefold volumes). The method of hydrolysis is quantitative, as verified by hydrolysis of a sample of authentic tetrahydrocortisone glucuronide.[1] Following hydrolysis, the samples were treated with trichloroacetic acid (2 ml. 10 per cent trichloroacetic acid added to 10 ml. of hydrolysate) and the precipitated protein removed by centrifugation. No appreciable steroid was lost as a result of this maneuver. After centrifugation, the clear supernatant fluid was extracted with dichloromethane. Reaction with the Porter-Silber phenylhydrazine-sulfuric acid reagent did not produce the pink chromogens reported by Migeon and co-workers (20). Urinary 17-OH-corticosteroids were also measured as Porter-Silber chromogen, with and without glucuronidase hydrolysis (total and free steroid, respectively) (25).

Procedures. Rate of hydrocortisone clearance was studied in 21 pregnant, 7 cirrhotic and 22 normal subjects in the following manner. Hydrocortisone (in the form of the water-soluble hemisuccinate or phosphate derivatives) in a dose of 100 mg. was injected intravenously over a period of two minutes. Serial blood specimens for free plasma 17-OH-corticosteroid determination were withdrawn at 40, 60, 120, 240 and 480 minutes, and in some instances, 20 minute samples were also taken. In certain of these subjects urine was also collected during the 480 minute period of study following hydrocortisone injection, and free and conjugated urinary 17-OH-corticosteroids were measured. In two pregnant and two normal individuals serial blood samples were withdrawn after hydrocortisone injection for the measurement of free and conjugated plasma 17-OH-corticosteroids.

Steroid disappearance rate was further studied by the administration of a reduced C21 metabolite of hydrocortisone. Tetrahydrocortisone, 75 mg. (approximately 1 mg. per Kg. body weight), was dissolved in 8 ml. of ethanol and administered intravenously in 50 to 75 ml. of isotonic saline over a period of five minutes. Subjects were five pregnant women and four normal women. Blood samples for measurement of free plasma 17-OH-corticosteroid levels were withdrawn at 20, 40, 60 and 90 minutes.

Response of plasma 17-OH-corticosteroids to corticotropin was studied in a manner previously described, in which steroid determinations were made on blood samples obtained before and after a four hour intravenous infusion of 25 I.U. adrenocorticotropic hormone (ACTH) (27). Subjects were nine pregnant women and six of the seven patients with Laennec's cirrhosis.

RESULTS

1. *Rate of hydrocortisone clearance from plasma during pregnancy*

Figure 1 shows the comparative rates of hydrocortisone clearance in 21 pregnant women near term and 13 normal women. The rate of hydrocortisone clearance is significantly slower in the pregnant subjects (difference in calculated slopes, pregnant *vs.* normal, $p < 0.01$). Average rate of decline of free plasma 17-OH-corticosteroid value is 15 per cent per hour in pregnancy, 27 per cent per hour in normal individuals. In a small series of women studied in the postpartum period the delayed clearance of hydrocortisone was found to persist for about six days, a period during which plasma 17-OH-corticosteroids remain elevated, although falling gradually toward normal (7, 10).

[1] Authentic tetrahydro E glucuronide was made available through the courtesy of Dr. Seymour Lieberman.

TABLE I

Free and conjugated plasma 17-OH-corticosteroid levels following the I.V. injection of 100 mg. hydrocortisone

Patients	Time (*mins.*)	Plasma 17-OH-corticosteroids, *μg.* % 20	40	60	120	240	480
Normal subjects (average of 2)	Free	195	155	152	94	52	22
	Conjugated*	22	27	15	14	12	0
Pregnant women (average of 2)	Free	204	170	167	139	107	74
	Conjugated	8	6	11	10	0	0

* In normal subjects, previous studies have tended to show higher values of 17-OH-corticosteroid glucuronides than are found in this study (9, 20, 23, 24). The discrepancy cannot be entirely accounted for. A possible explanation is the fact that in this investigation the injected steroid was the succinate or phosphate derivative of hydrocortisone. The earlier studies were carried out with hydrocortisone alcohol (labeled or unlabeled) (9, 20, 23, 24). The derivatives may be metabolized differently than the free steroid. Some support for this supposition may be derived from the fact that in the present study a greater absolute and relative amount of free urinary corticosteroid was observed after hydrocortisone hemisuccinate or phosphate administration than was reported by Migeon and associates, who employed isotopic hydrocortisone alcohol (9) (see Table II).

Table I shows hydrocortisone clearance and the appearance in plasma of conjugated 17-OH-corticosteroids in two normal and two pregnant subjects. The delayed clearance of hydrocortisone in the pregnant women is accompanied by subnormal levels of 17-OH-corticosteroid glucuronides in simultaneous blood samples. The data confirm the observations of Migeon and associates (9, 20).

Table II shows the results of steroid measurements in urine following hydrocortisone injection. It will be seen that the average quantity of free 17-OH-corticosteroid found in the urine of six pregnant women (8.3 mg. per eight hours) is much larger than that found in urine of six normal subjects (4.7 mg. per eight hours). This finding also confirms the earlier studies (9, 20). The nearly equal values for conjugated urinary 17-OH-corticosteroids (16.3 and 14.6 mg. per eight hours) in pregnant and normal subjects may be in part due to measurement of a nonspecific chromogen which appears in the urine of pregnant women after enzymatic hydrolysis. Conjugated 17-OH-corticosteroids in pregnancy urine are somewhat higher than normal by this technique (10). It is of interest that Migeon and co-workers, using 4-C^{14}-cortisol, found only moderate reductions in urinary excretion of corticosteroid glucuronides during pregnancy (9).

TABLE II

Free and conjugated urinary 17-OH-corticosteroids during the eight hour period following the I.V. injection of 100 mg. hydrocortisone

	No. of subjects	Average value of urinary steroids: Conjugated	Free	Ratio of conjugated to free steroid
		mg./8 hrs.		
Normal subjects	6	14.6	4.7	3.1 : 1
Pregnant women	6	16.3	8.3	1.97 : 1

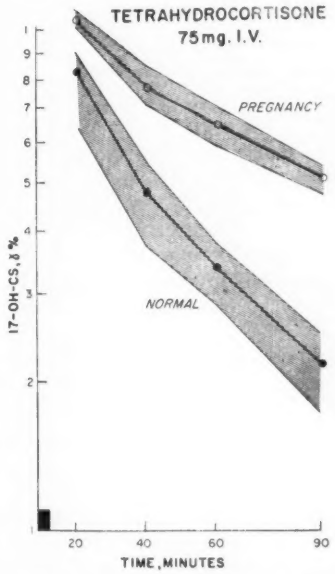

FIG. 2. SEMILOGARITHMIC PLOT OF THE RATE OF FALL OF PLASMA 17-OH-CORTICOSTEROID LEVELS AFTER INTRAVENOUS INJECTION OF TETRAHYDROCORTISONE, 75 MG.

Solid lines indicate averages for the five pregnant and four normal subjects; shaded areas indicate the extreme limits of variation for the two groups. Average per cent decrease of plasma 17-OH-corticosteroid level (per hour) for pregnant women is significantly less than that for normal subjects at the 5 per cent level.

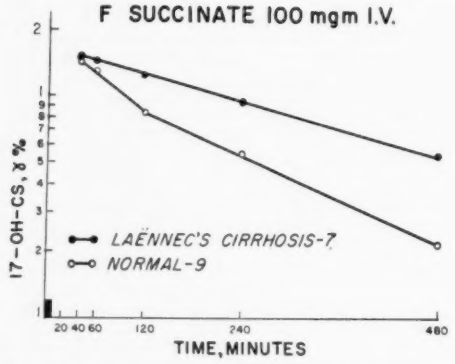

FIG. 3. SEMILOGARITHMIC PLOT OF RATE OF FALL OF PLASMA 17-OH-CORTICOSTEROID LEVELS IN SEVEN PATIENTS WITH CIRRHOSIS AND NINE NORMAL SUBJECTS

Lines represent average rates of decline.

2. Rate of tetrahydrocortisone clearance from plasma in pregnancy

Figure 2 shows that the average clearance of tetrahydrocortisone is slower in pregnant than in normal women. The slopes of the lines describing rate of decline of steroid level in the two groups differ significantly ($p < 0.05$). "Half-life" is about 75 minutes in the pregnant women and 48 minutes in the normal individuals. In patients with Laennec's cirrhosis tetrahydrocortisone disappearance rate has been reported to be normal (23, 24).

3. Rate of hydrocortisone clearance from plasma in cirrhosis

Figure 3 compares hydrocortisone clearance rates in patients with cirrhosis and normal subjects. The finding of a delayed clearance rate in the cirrhotic patients is in agreement with previous observations (22–24).

4. Response of plasma 17-OH-corticosteroid levels to ACTH in pregnancy and cirrhosis

Figure 4 shows the apparently exaggerated response to ACTH of plasma (free) 17-OH-corticosteroid levels in the third trimester of pregnancy (27). Plasma steroid levels are above normal before and after ACTH administration, and the average increment is significantly greater than normal ($p < 0.01$) (28). This finding is not in agreement with the observation of Migeon and associates who found essentially normal plasma 17-OH-corticosteroid response to ACTH near term (9).

Figure 5 shows that the plasma steroid response to corticotropin in the six patients with cirrhosis is normal or only slightly exaggerated. Average pre- and post-ACTH levels, 18 and 47 μg. per 100 ml., respectively, are within the normal limits (29).

DISCUSSION

A delay in the rate of clearance of hydrocortisone from plasma of pregnant women near term appears to be well established by earlier work (9, 20) and by the present study. This observation raises two points for discussion: *1*) the problem of whether or not the slower rate of hydrocortisone clearance completely explains the elevated plasma hydrocortisone levels found in late pregnancy, and *2*) the mechanism responsible for the delay in hydrocortisone clearance.

If the assumption is made that the elevated plasma hydrocortisone level of late pregnancy is due entirely to its slower rate of disappearance from plasma, it might be reasonable to expect elevated plasma hydrocortisone levels in other clinical conditions in which hydrocortisone clearance from plasma is delayed. The data of these and earlier studies (22–24) indicate that the levels of plasma 17-OH-corticosteroids in one such condition, Laennec's cirrhosis, are normal despite a definitely reduced rate of hydrocortisone clearance. Furthermore, in contrast to the apparently excessive response of free plasma 17-OH-corticosteroid levels to ACTH in pregnancy [found to be normal by Migeon and co-workers (9)], steroid response to corticotropin appears to be essentially normal in cirrhosis. Several explanations might be offered for these differences between the findings in pregnancy and cirrhosis. First, one might suppose that adrenal secretory rate is reduced in cirrhosis, and that a moderate decrease in hydrocortisone production is obscured by a normal plasma level which in turn depends upon a delay in clearance of hydrocortisone from plasma. Such an idea receives support from the work of Samuels, Brown, Eik-Nes, Tyler and Dominguez (30). The conclusion drawn by these workers that adrenal secretory rate of hydrocortisone is reduced by about 40 per cent in cirrhosis was based on calculations derived from the slope of plasma hydrocortisone response to ACTH and

the slope of hydrocortisone disappearance rate from plasma after a loading dose of this steroid. While further experiments may prove this conclusion to be correct, it is fair to state that the method of computation employed constitutes an indirect approach to the question which can perhaps be more directly attacked by newer methods using isotopically labeled steroids (31).

Second, it might be assumed that hydrocortisone is more firmly bound to plasma proteins in pregnant women than in normal or cirrhotic subjects. This assumption could explain the elevated plasma hydrocortisone level of pregnancy and the delayed hydrocortisone disappearance rate. However, Daughaday has reported that there is no increase in corticosteroid binding during gestation (32).

Third, it is conceivable that there is a derangement in one of the homeostatic mechanisms which normally regulates the rate of ACTH release from the anterior pituitary. It is well known that the level of circulating hydrocortisone is inversely proportional to the rate of corticotropin release (33). If this regulatory mechanism were normal in pregnancy, one would expect that a higher plasma hydrocortisone level would act to inhibit ACTH release, and that, lacking the stimulation of corticotropin, the adrenal cortex would secrete less hydrocortisone, resulting finally in a normal level of plasma hydrocortisone. Such a sequence of events might explain the findings in cirrhosis.

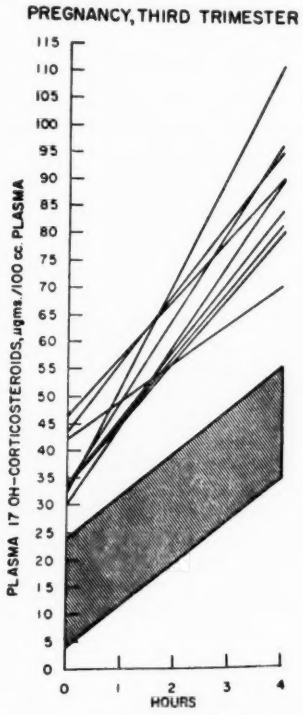

FIG. 4. RESPONSE OF PLASMA 17-OH-CORTICOSTEROID LEVELS TO ACTH IN PREGNANCY (NINE WOMEN)

Solid lines indicate responses of the pregnant women, shaded area shows extreme limits of variation in 40 normal subjects (29).

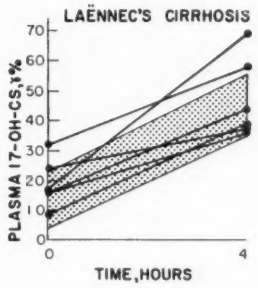

FIG. 5. RESPONSE OF PLASMA 17-OH-CORTICOSTEROID LEVELS IN SIX PATIENTS WITH CIRRHOSIS (SOLID LINES) AS COMPARED TO NORMAL RESPONSE (SHADED AREA)

The postulate of an altered homeostatic control of ACTH release in pregnancy may present a problem susceptible to investigation. Previous studies have shown that administration of 17 alpha, 21 - dihydroxy - delta^{1,4} - pregnadiene - 3,20 - dione (prednisone) is capable of suppressing the apparently exaggerated response of plasma 17-OH-corticosteroids to ACTH in pregnancy (29), but the degree of suppression is less than that brought about by prednisone in normal subjects. These findings suggest that the pituitary during pregnancy can be influenced by circulating glucocorticoid levels, but that it is perhaps less sensitive than normal to this influence.

Concerning the mechanism of the delayed hydrocortisone clearance rate in pregnancy, the data on tetrahydrocortisone clearance seem pertinent. Peterson and Englert and their respective associates (23, 24) have reported that the tetrahydrocortisone clearance rate from plasma is normal in patients with cirrhosis. On the basis of these data, it has been postulated that the delayed disappearance rate of hydrocortisone in cirrhosis is due to a defect in hepatic reduction of the steroid nucleus (*i.e.*, reduction of hydrocortisone to tetrahydrocortisone and tetrahydrocortisol) and not due

to a defect in hepatic conjugation of the reduced (tetrahydro) compounds with glucuronic acid (24).[2] In this connection, Isselbacher and Axelrod showed that reduction of ring A of the steroid nucleus is an essential prerequisite for steroid glucuronide formation in liver (35). The finding in the present study of a delayed disappearance rate of injected tetrahydrocortisone in pregnancy differs from the observations in cirrhosis, but is similar to the finding of delayed tetrahydrocortisone clearance in a single patient with congenital nonobstructive, nonhemolytic jaundice (36). This condition, in which resting free plasma 17-OH-corticosteroid levels are normal, is presumably characterized by defective hepatic formation of various glucuronides (36). The delayed tetrahydrocortisone clearance observed in pregnancy might be explained in a number of ways, but whatever the correct explanation, the mechanism would appear to be different from that operative in cirrhosis. Although several pathways of hydrocortisone metabolism may be involved [*e.g.*, hydroxylation at the C20 position, as demonstrated by Fukushima, Leeds, Bradlow, Kritchevsky, Stokem and Gallagher (37)], it is possible that the delayed hydrocortisone clearance rate in pregnancy may be related to defective conjugation of tetrahydrocortisone (and by extension, of tetrahydrocortisol) with glucuronic acid rather than altered reduction of hydrocortisone to its tetrahydro derivatives. Whether or not the delayed tetrahydrocortisone clearance rate can be explained by changes in binding of this steroid by plasma proteins is a question that awaits investigation.

In conclusion, it seems likely that delayed hydrocortisone clearance may partially explain the high plasma hydrocortisone level of late pregnancy. It seems improbable that this delayed hydrocortisone disappearance rate is the complete explanation, since delayed hydrocortisone clearance in cirrhosis is not accompanied by high plasma 17-OH-corticosteroid levels. It is possible that in pregnancy there exists a change in the homeostatic mechanism whereby hydrocortisone and corticotropin influence the rate of release of one another.

[2] Cohn and Bondy were able to detect only trace quantities of tetrahydrocortisone and tetrahydrocortisol glucuronides in this disease, but found significant amounts of unconjugated tetrahydrocortisone and tetrahydrocortisol (34). These authors suggested that in cirrhosis there may be a delayed formation of steroid glucuronide in addition to a reduced rate of reduction of the delta^4-3-ketone in ring A (34).

SUMMARY

1. A delay in the clearance rate of hydrocortisone from plasma in late pregnancy and in cirrhosis of the liver is confirmed.

2. Plasma 17-OH-corticosteroid levels and their response to adrenocorticotropic hormone (ACTH) are greater than normal in late pregnancy, but normal in cirrhosis.

3. Tetrahydrocortisone clearance rate is delayed in pregnancy and reportedly normal in cirrhosis, suggesting that the delay in hydrocortisone clearance may have a different basis in the two conditions.

4. It is suggested that the elevated plasma 17-OH-corticosteroid level in late pregnancy cannot be entirely accounted for by a delay in the rate of hydrocortisone clearance from plasma.

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MYOCARDIAL RESPONSE TO ACETYL STROPHANTHIDIN IN CONGESTIVE HEART FAILURE: A STUDY OF ELECTROLYTES AND CARBOHYDRATE SUBSTRATES *

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The concept of a defect in the conversion of chemical energy to work as the cause of low output heart failure has been based on the finding of normal myocardial extraction of substrate and oxygen in this clinical state (1, 2). However, the alternative view, that there is failure in transformation of the energy of substrate oxidations to a biologically useful form, cannot be excluded (3). Of particular interest, as regards the former theory, is the problem of evaluating those physical-chemical factors which initiate or contribute to the alteration of myocardial protein in heart failure.

A consideration of the role of changes in intracellular ionic content in the regulation of cardiac performance has been stimulated by finding an intimate relation between these parameters in the staircase phenomenon (4). Other isolated muscle work has shown that changes in electrolyte concentration at the superficial level of the cell can affect contractile strength of muscle before the ion penetrates the interior of the muscle (5, 6).

Although there is at present no adequate methodology for precise study of these phenomena in the human heart *in vivo*, it was considered of importance to seek information concerning the myocardial transfer of ions in the limited circumstance of drug-induced recovery from cardiac failure. In a prior report (7), data were presented which indicated the facility with which acetyl strophanthidin mobilized electrolytes in the myocardium of the intact dog, its abrupt course of action enabling detection of these changes.

METHODS

The design of this study was such as to rely on the assumption that the coronary arteriovenous differences of electrolytes and metabolites could be validly accepted as a measure of their net uptake by, or release from, the myocardium. Although the transcapillary exchange rates of an ion, such as potassium, are blood flow dependent (8), an estimation of the effects of altered blood flow *per se* upon measured arteriovenous differences appeared in order. In the intact human, two procedures which usually increase coronary blood flow, exercise (9) and hypoxia (10), were chosen to study coronary arteriovenous differences of potassium. Five minutes of exercise produced no significant change in six patients. The mean of the planimetered A-V difference areas for potassium was $+0.01 \pm 0.08$ square inches in the control and $+0.06 \pm 0.20$ in the experimental period. Similar results were obtained with hypoxia, induced by 10 per cent oxygen inhalation for 25 minutes in five patients. The mean control value was $+0.06 \pm 0.25$ square inch and the hypoxia value, $+0.27 \pm 0.23$. These findings are in agreement with those of Driscol and Berne (11), in which coronary blood in the dog was increased up to 100 per cent by varied procedures without significant change in the potassium arteriovenous differences.

The effects of digitalis preparations on coronary blood flow in the human have not been elaborated except for strophanthus, which produced no change (12). If one assumes that acetyl strophanthidin behaves similarly or increases coronary flow within the limits of the above experiments, then inferences may be drawn in regard to net movement of ions. An unexpected decrease in coronary blood flow should reveal increases in extraction of oxygen and cation, events which, as detailed below, did not occur.

Eighteen patients were chosen from the emergency room service, who presented themselves with the usual

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TABLE I

Sequential hemodynamic response to acetyl strophanthidin, 1.1 mg., in five patients with low output heart failure

Patient Age, race, sex Diagnosis B.S.A. in M.²	Time	Pulse	Mean arterial pressure	Oxygen consumption	Systemic A-V oxygen difference	Cardiac output	Stroke output	Stroke work	Mean pressures: Pulmonary capillary	Mean pressures: Pulmonary artery
	min.		*mm. Hg*	*ml./min.*	*ml./L.*	*L./min.*	*ml./beat*	*Gm./M.*	*mm. Hg*	
W. D.	0	120	130	275	85.4	3.22	26.8	34.8	28	44
66, W. M.	5	90		335	84.9	3.95	43.8		40	50
HCVD	15	70	142	265	63.6	4.17	59.7	84.8	37	45
1.78	25	78	120	270	68.3	3.95	50.6	60.7	30	38
M. B.	0	92	176	255	91.5	2.46	26.7	47.0	30	57
38, N. F.	5	92	170	343	81.8	4.19	45.5	77.4		64
HCVD	15	91	182	340	69.1	4.92	54.1	98.5		62
1.95	25	94	170	267	57.3	4.66	49.5	94.2		55
J. B.	0	91	116	303	93.0	3.26	35.8	41.5	28	49
47, N. M.	5	85	132	303	101.3	3.96	45.4	60.0	31	56
ASHD	15	86	122	303	76.6	3.96	46.0	56.1	40	53
2.14	25	86	106	293	73.4	3.99	46.4	49.3	33	54
E. S.	0	115	164	281	104.3	2.69	23.4	38.4	37	68
39, N. F.	5	115	202	360	124.2	2.90	25.2	50.9		70
HCVD	15	103	200	306	93.4	3.28	31.8	63.6		70
1.83	25	102	180	307	85.5	3.59	35.2	63.4		66
J. B.	0	71	123	212	62.7	3.38	47.6	58.5	25	32
49, N. M.	5	68	137	230	55.5	4.14	60.9	83.4		31
ASHD	15	67	111	220	53.6	4.10	61.2	67.9		21
1.77	25	68	100	227	55.7	4.08	60.0	60.0	16	24
Mean	0	98	141	265	87.4	3.00	32.06	44.0		50
values	5	90	160	315	89.5	3.83	44.16	67.9		54
	15	83	151	286	71.3	4.09	50.56	74.2		50
	25	86	135	272	68.0	4.05	48.34	65.5		47

symptoms and evidence of left-sided or left- and right-sided heart failure. X-ray evidence of pulmonary congestion and cardiac enlargement was present in all. There was no evidence of primary valvular heart disease. The clinical types included hypertensive, arteriosclerotic and unknown etiologies. No patient had received digitalis therapy in the preceding three weeks, nor diuretics for the preceding four days. Oxygen, in the semi-Fowler position, was the only therapy received prior to the period of study, which was performed within 18 hours after admission. Nembutal®, 1.5 gr. intramuscularly, was administered about two hours before the collection of the first samples. With catheters in the coronary sinus and right atrium, and a Cournand needle in the brachial artery, simultaneous blood sampling was performed.

Acetyl strophanthidin,[1] 1.1 mg., was injected into the right atrium over a period of one and one-half minutes. The mid-point of injection was considered zero time. Since the hemodynamic response to this drug, in a group of five patients in which serial Fick cardiac outputs were obtained, reached maximum by 15 minutes (Table I), the cardiac output was determined in the experimental group at this time. The control determination was five minutes before infusion, and, at the same time, simultaneous sampling of arterial and coronary sinus blood for control A-V difference of oxygen, carbon dioxide, glucose, lactate and pyruvate was performed. Sampling for these substances was repeated at 5 to 7 minutes and at 15 minutes after injection of strophanthidin. Paired samples for electrolyte determinations were drawn from the brachial artery and coronary sinus at about 14 and 4 minutes before giving the drug, and approximately at 2, 4, 7, 10, 14, 20 and 25 minutes afterward.

Phasic and mean arterial pressures were obtained at 5 minute intervals with a Sanborn electromanometer and recorded on the Sanborn polyviso. Stroke work against pressure was calculated as the product of the mean femoral artery pressure and stroke output, and expressed in gram-meters (13). The electrocardiogram (Lead V_6 or V_7) was recorded on the polyviso just prior to giving the drug and at 1 minute intervals for 15 minutes; thereafter, at 5 minute intervals.

Oxygen consumption was determined by analysis of expired air and room air for oxygen, on a Pauling oxygen analyzer. The blood gas analyses were carried out in duplicate by the method of Van Slyke and Neil. Determinations of glucose, lactic acid and pyruvic acid, performed in 11 patients of the group, were done by the methods of Somogyi (14), Barker and Summerson (15), and Friedemann and Haugen (16), respectively. Sodium

[1] Kindly supplied by Dr. Kenneth G. Kohlstaedt, Medical Department, Eli Lilly and Company, Indianapolis, Ind.

TABLE II

Findings before and after 1.1 mg. of acetyl strophanthidin in 18 patients with low output heart failure

Patient Age, race, sex Diagnosis	Time	Stroke output	Stroke work	O_2 BA–CS	CO_2 (CS–BA)	Hematocrit BA	Hematocrit CS	Electrocardiogram Heart rate	QT	ST	T wave	Rhythm
	min.	*ml./beat*	*Gm./M*	*ml./100 ml.*	*ml./100 ml.*							
J. B.	0	61.9	66.9	11.73	5.75	40	41	78	0.43	ISO†	Upright	N‡
70, N. M.	5					40	41	82	0.40	S§	S	S
ASHD*	15	96.1	113.4	11.39	9.03	39	40	80	0.40	S	S	S
M. P.	0	41.9	53.6	13.29	9.78	38	39	76	0.38	ISO	Inverted	Few PVC
56, W. M.	5					38	39	70	0.38	Straight	S	S
HCVD‖	15	75.5	99.7	12.45	17.73	37	38	66	0.38	Straight	S	S
W. C.	0	80.9	88.2	12.95	9.75	43	44	80	0.37	ISO	ISO	N
48, N. M.	5			13.01	10.58	44	44	72	0.38	S	S	S
ASHD	15	111.5	131.6	12.90	7.30	43	44	74	0.38	S	S	S
D. M.	0	72.1	77.9					83	0.39	ISO	Inverted	N
42, W. M.	5							82	0.38	S	S	S
HCVD	15	86.9	117.3					80	0.38	S	S	S
B. L.	0	75.6	128.5	7.39	5.43	26	26	91	0.42	ISO	Upright	N
39, W. F.	5					26	26	90	0.41	S	S	S
HCVD/An.	15	87.4	162.6	6.75	4.72	26	26	86	0.41	S	S	S
J. S.	0	37.3	49.2	12.25	8.36			72	0.38	ISO	Upright	N
64, W. M.	5			11.41				63	0.36	Shortened	S	S
HCVD	15	54.8	72.3	11.48	9.38			78	0.38	Shortened	S	S
J. T.	0	25.7	25.2	15.62	13.81	50	50	90	0.32	ISO	Upright	Few PVC¶
45, N. M.	5					50	50	106	0.26	Shortened	Diphasic	N
Idiopathic	15	38.9	43.5	15.97	10.80	49	49	72	0.28	Shortened	Diphasic	N
G. A.	0	25.8	30.2	13.16	15.15	46	46	96	0.34	ISO	Upright	N
38, N. M.	5					45	46	94	0.32	S	S	N
HCVD	15	33.3	44.6	15.4	15.71	45	46	92	0.32	S	S	N
J. T.	0	61.5	96	11.92	10.60	45	45	86	0.38	ISO	Inverted	N
50, W. M.	5					44	45	83	0.39	S	S	N
HCVD	15	66	112	13.01	9.36	45	45	73	0.38	S	S	N
W. C.	0	40.4	32.3	12.00	8.83	40	41	98	0.34	ISO	ISO	N
41, N. M.	5					40	40	72	0.36	S	S	N
Idiopathic	15	46.7	43.9	11.39	9.94	39	40	90	0.34	S	S	N
E. L.	0	28.3	47.0	14.81	11.76	46	46	115	0.33	ISO	Inverted	N
70, W. M.	5					45	45	111	0.30	S	Deeper	Few PVC
HCVD	15	33.2	56.4	13.60	11.16	44	44	120	0.31	S	Deeper	N
E. R.	0	63.8	81.7	12.39	8.27	41	42	77	0.42	ISO	Upright	N
56, N. M.	5			12.30		41	41	72	0.38	S	Diminish.	N
HCVD	15	68.0	87.0	12.36	8.24	41	42	77	0.38	S	Diminish.	N
P. T.	0	57.2	74.4	11.26		46	46	83	0.42	ISO	Diphasic	N
39, N. M.	5					46	46	86	0.41	S	S	N
Myocard.	15	69.1	71.9	11.36		46	46	79	0.41	S	S	N
H. M.	0	58.2	60.5	12.85	9.62	50	50	100	0.34	ISO	Upright	N
40, N. M.	5					50	50	92	0.32	S	S	N
Idiopathic	15	54.9	68.1	10.97	4.11	50	50	100	0.32	S	S	N
H. W.	0	47	69.6	12.04	11.13	47	48	83	0.34	ISO	Inverted	N
39, N. M.	5			13.37	10.32	48	47	81	0.32	S	S	N
HCVD	15	55.5	60.5	12.46	8.84	47	47	83	0.32	S	S	N

* ASHD, arteriosclerotic heart disease.
† ISO, isoelectric.
‡ N, normal.
§ S, same as control period.
‖ HCVD, hypertensive cardiovascular disease.
¶ PVC, premature ventricular contractions.

TABLE II—*Continued*

Patient Age, race, sex Diagnosis	Time	Stroke output	Stroke work	O_2 BA–CS	CO_2 (CS–BA)	Hematocrit BA	Hematocrit CS	Electrocardiogram Heart rate	QT	ST	T wave	Rhythm
	min.	*ml./beat*	*Gm./M.*	*ml./100 ml.*	*ml./100 ml.*							
L. B.	0	59.1	68.6	10.66	7.39	37	37	84	0.38	ISO	Diphasic	N
50, N. M.	5					36	37	66	0.36	S	S	N
ASHD	15	62.3	52.3	10 49	7.38	36	37	75	0.37	S	S	N
E. L.	0	106.4	173.4	8.80	6.30	30	30	75	0.44	ISO	Upright	Few PVC
51, W. M.	5			8.83	6.07	30	30	71	0.40	S	Diphasic	S
HCVD/An.	15	81.2	139.7	8.25	6.64	29	29	76	0.42	S	Upright	S
D. H.	0	98.6	138.0	7.78	5.38	48	48	86	0.40	ISO	Upright	N
63, W. M.	5					47	47	84	0.40	S	S	N
Idiopathic	15	65.8	55.0	11.91	9.94	47	46	84	0.40	S	S	N
Mean	0			11.82	9.21			86				
values	5							82				
	15			11.89	9.39			83				

and potassium determinations were done in duplicate, with less than 2 per cent error on a Beckman flame photometer, using samples which showed no visible hemolysis. Calcium analysis was done in seven patients by the versene titration method of Zak, Hindman and Fisher (17), with an error of determination less than 3 per cent.

Since the plotting of electrolyte concentrations on linear graph paper would seem to give a more complete picture of the changes occurring than just a comparison of maximum change in arteriovenous differences, identical time periods have been planimetered for comparison (Figure 1). The nadir of the relatively rapid fall in coronary sinus potassium concentration following the maximum response was chosen as the end of an arbitrary period (Period II) during which the maximum response to the drug occurred. The area under the curve was planimetered from the first minute to the end of this period at 10 to 13 minutes. An identical time (Period I) prior to drug injection was measured for comparison in each case, and the same duration was used for Period III, when the maximum hemodynamic response had been attained or was waning. Standard methods were used in applying the test for statistical significance of the observed changes (18).

RESULTS

The general hemodynamic response to 1.1 mg. of intravenous acetyl strophanthidin in five patients is tabulated in Table I. As judged by the per cent increase in stroke work, the major effect had been attained by 5 minutes, and, in two instances, this was the maximum effect observed. The rapid changes during the time of the drug's action make the Fick method less accurate, but the close approximation of the oxygen consumption and pulse rate in two successive determinations, at 5 and 15 minutes for M.B. and J.B., and 15 and 25 minutes for W.D., E.S. and J.B., indicate sufficient reliability for hemodynamic interpretations. The use of the 15 minute mark to qualitatively assess the inotropic response in the group studied for myocardial metabolism seems warranted by these data. The transient paradoxic rise in pulmonary arterial pressures while the cardiac output is rising probably resulted from a rapid displacement of blood from the periphery at this time.

The patients in Table II have been arranged in decreasing order of stroke output and work response. The fact that 8 of 18 failed to react in a therapeutic way may represent a compounding of several factors: the brief period of action of this

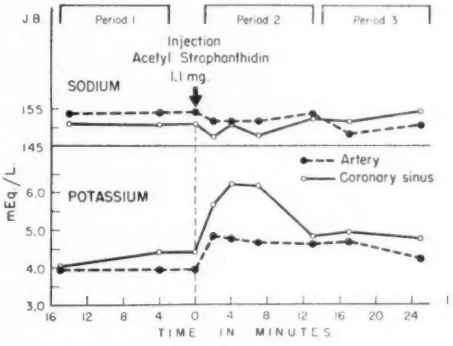

FIG. 1. A REPRESENTATIVE ELECTROLYTE RESPONSE TO ACETYL STROPHANTHIDIN

The equal time periods delimit the time-concentration areas which were planimetered for comparisons. These areas are considered positive when the arterial concentration exceeds the venous, and negative when the venous exceeds the arterial.

TABLE III

Arterial levels and myocardial extraction of carbohydrate substrates

	Lactate						Pyruvate				Glucose					
	Control		7 minutes		15 minutes		Control		7 minutes		15 minutes		Control		7 minutes		15 minutes	
	B.A.	A-V	B.A.	A-V	B.A.	A-V	B.A.	A-V	B.A.	A-V	B.A.	A-V	B.A.	A-V	B.A.	A-V	B.A.	A-V
M. P.	8.43	+2.40	13.32	+3.10	8.64	+1.51	1.15	+0.30	0.95	+0.29	0.98	+0.24	74.0	+1.5	77.0	0.0	68.5	−16.0
J. S.	5.37	+2.07	5.46	+2.01	5.34	+0.12	1.19	+0.14	1.02	+0.06	1.09	+0.11	81.0	−0.5	80.5	+4.0	78.5	+ 0.5
J. T.	15.80	+1.80			13.90	+0.60	1.57	+0.16			1.37	+0.15	90.5	+9.0			89.0	0.0
J. T.	14.00	+2.40			11.00	−2.20	1.63	+0.06			1.44	+0.17	84.1	+1.2			85.0	+++4.9
W. C.	12.60	+3.97	9.66	+3.36	9.87	+4.07	1.73	+0.62	1.36	+0.54	1.34	+0.50	117.0	+1.0	116.0	+2.0	99.0	8.0
E. L.	10.77	+2.37	11.73	+1.32	10.71	+0.42	1.52	+0.13	1.80	+0.56	1.47	+0.35	110.5	+6.5	100.0	+2.5	100.0	7.5
H. M.	6.72	+2.34	6.00	+2.55	4.41	+1.59	1.24	+0.41	1.03	+0.29	1.13	+0.34	101.0	+5.0	97.0	−1.0	104.0	+10.5
H. W.	9.98	+4.10	8.19	+3.75	5.37	+1.01	1.27	+0.03	1.69	+0.43	1.36	+0.10	91.0	+3.0	87.0	+1.0	93.0	− 3.0
L. B.	7.86	+2.28	7.80	+2.22	8.04	+3.75	1.04	+0.34	0.91	+0.26	0.94	+0.29	96.0	−1.5	99.0	+2.5	98.0	4.0
E. L.	4.83	+2.37	3.36	+2.46	2.52	−0.78	0.71	+0.17	0.63	+0.28	0.58	+0.18	85.0	+6.0	86.0	+7.0	81.0	+++ 5.0
D. H.	4.05	+0.12	5.10	+1.05	4.17	+1.27	1.62	+0.10	1.55	+0.08	1.60	+0.19	101.0	0.0	105.5	+4.0	106.5	5.5
E. R.													76.5	+4.0	71.5	−1.5	75.5	− 0.5
Mean	9.05	+2.38	7.85	+2.42	7.63	+0.84	1.33	+0.223	1.21	+0.31	1.21	+0.24	92.3	+2.9	92.0	+2.1	89.8	+2.16
S. D.	±3.89		±4.09		±3.52	±1.99	±0.31				±0.40							
S. E.	±1.17		±1.03		±1.06	±0.63	±0.09				±0.09							
p					<0.02*	<0.05*					<0.01*							
						>0.02												

* Comparison with control.

digitalis preparation, the degree of myocardial disease, and the blood loss necessitated by the sampling procedures.

The myocardial extraction of oxygen was not significantly changed by strophanthidin from the mean control level of 11.82 ml. per 100 ml., a value slightly higher than the 10.3 ml. per 100 ml. in normals for this laboratory (19), which becomes more elevated if the anemic patients are excluded. The carbon dioxide production response showed wide variation with no significant pattern for the group. Likewise, there were no gross changes in myocardial water transfer as measured by the rough index of hematocrit. While the chronotropic response was small but definite in most instances, the electrocardiographic response was not indicative of digitalization except in three instances, M.P., J.S. and J.T., where minimal ST segment changes occurred. In only one patient, a 70 year old male, was there evidence of a toxic effect from strophanthidin, with unifocal premature ventricular beats, lasting, transiently, for two minutes.

The mean arterial levels of the three carbohydrate substrates measured were all elevated during the period of untreated heart failure (Table III), but only lactate and pyruvate declined significantly toward the normal levels after treatment (19). The extraction of these substances appeared to be maintained normally by the failing heart, although this was probably dependent upon the elevated arterial levels.

The extraction of pyruvate and glucose was not altered after strophanthidin, but lactate extraction, although unchanged at 7 minutes, was substantially diminished at 15 minutes. The p value for this change was slightly higher than 0.02 but less than 0.05, thus bearing a fair probability of significance.

As representatively depicted in Figure 1, acetyl strophanthidin precipitated a rapid movement of potassium from the heart. This was true for all but one of the group of 18 (Table IV), a patient with the clinical criteria of myocarditis who did not respond to strophanthidin therapeutically and who was poorly manageable on a clinical basis.

The maximum negative potassium A-V difference usually occurred at four minutes after infusion, and measurement of Period II, as designated

TABLE IV

Myocardial arteriovenous differences of potassium and sodium before and after acetyl strophanthidin

	Potassium (*mEq./L.*)									Planimetered area in sq. in.		
	Control			Maximum			Recovery*					
Patient	B.A.	C.S.	Diff.	B.A.	C.S.	Diff.	B.A.	C.S.	Diff.	I	II	III
J. B.	3.94	4.42	−0.48	4.78	6.22†	−1.44	4.65	4.85	−0.20	−0.77	−3.10	−1.1
M. P.	3.76	3.42	+0.34	4.06	4.48†	−0.42	3.79	3.66	+0.13	+0.50	−0.34	+0.16
W. C.	4.26	4.23	+0.03	4.64	4.98†	−0.34	Still—A-V at 25 min.			+0.04	−0.75	−0.77
D. M.	4.20	4.30	−0.10	4.90	5.70†	−0.80	5.00	4.90	+0.10	−0.60	−1.64	−0.12
B. L.	4.64	4.37	+0.27	4.66	5.04†	−0.38	Still—A-V at 25 min.			+0.36	−0.33	−0.38
J. S.	5.37	5.31	+0.06	6.09	6.51†	−0.39	5.79	5.56	+0.23	+0.06	−0.58	+1.3
J. T.	4.56	4.65	−0.09	5.19	5.76†	−0.57	5.20	5.42	−0.22	+1.17	−0.94	
G. A.	4.39	4.42	−0.03	4.91	5.88‡	−0.97	4.72	4.78	−0.06	−0.23	−1.66	−0.10
J. T.	3.79	3.20	+0.59	4.32	4.80‡	−0.48	3.60	3.60	0.00	+1.12	+0.16	−0.10
W. C.	4.85	4.81	+0.04	4.79	5.41‡	−0.62	Still—A-V at 25 min.			−0.02	−1.0	−2.31
E. L.	3.89	3.65	+0.25	3.75	4.70‡	−0.95	4.05	3.98	+0.07	+0.51	−1.19	−0.56
E. R.	4.34	4.51	−0.17	4.51	5.68‡	−1.17	4.75	4.71	+0.04	−0.40	−0.98	+0.00
P. T.	4.25	4.50	−0.25	4.75	4.76†	−0.01	4.78	4.79	−0.01	−0.08	+0.12	+0.44
H. M.	5.24	5.34	−0.10	5.60	6.50†	−0.91	5.89	5.92	−0.03	−0.25	−1.15	+0.60
H. W.	4.58	4.47	+0.11	4.95	5.58†	−0.63	4.54	4.41	+0.13	+0.10	−1.33	−1.38
L. B.	4.65	4.40	+0.25	4.97	5.29†	−0.59	4.66	4.74	−0.08	+0.30	−0.46	+0.36
E. L.	3.85	3.83	+0.02	4.04	4.52†	−0.48	4.01	4.16	−0.15	−0.09	−1.09	−0.48
D. H.	4.68	4.59	+0.08	5.09	5.30‡	−0.21	4.99	4.75	+0.24	+0.03	−0.24	−0.33
Mean	4.40		+0.05	4.77		−0.63	4.69		+0.01	+0.097	−0.94	−0.45
S. D.	±0.49			±0.768			±0.666			±0.51	±0.74	±0.76
S. E.	±0.12			±0.181			±0.178			±0.12	±0.17	±0.18
t				5.07							7.2	2.5
p				<0.001‖							<0.001‖	<0.05%‖

	Sodium (*mEq./L.*)									Planimetered area in sq. in.		
	Control			Maximum			Recovery*					
Patient	B.A.	C.S.	Diff.	B.A.	C.S.	Diff.	B.A.	C.S.	Diff.	I	II	III
J. B.	154	151	+3.0	152	151†	+1.0	154	152	+2.0	+1.10	+0.91	−0.80
M. P.	134	135	−1.0	137	133§	+4.0	135	135	0.0	−0 03	+0.64	−0.45
W. C.	152	151	+1.0	152	146‡	+6.0	150	151	−1.0	−0.09	+0.35	−0.97
D. M.												
B. L.	131	134	−3.0	131	131†	0.0	127	131	−4.0	−0.9	−0.9	−1.60
J. S.												
J. T.	152	150	+2.0	147	153†	−6.0	Still—A-V at 15 min.			−1.0	−1.31	
G. A.	137	140	−3.0	137	138†	−1.0	139	139	0.0	−1.4	+0.20	−0.10
J. T.	153	153	0.0	158	153§	+5.0	154	152	+2.0	0.0	+0.81	−0.51
W. C.	157	158	−1.0	157	149†	+8.0	154	153	+1.0	−0.01	+0.77	−0.80
E. L.	134	134	0.0	133	131†	+2.0	131	131	0.0	−0.08	+0.09	−0.02
E. R.	152	151	+1.0	149	151†	−2.0	153	148	+5.0	−0.08	+0.06	+0.21
P. T.	138	139	−1.0	137	135†	+2.0	136	135	+1.0	−0.24	+0.31	+0.27
H. M.	152	157	−5.0	155	156§	−1.0	160	154	+6.0	−1.13	−0.93	+1.18
H. W.	157	152	+5.0	154	147†	+7.0	149	148	+1.0	+0.92	+0.90	−1.66
L. B.	151	152	−1.0	150	150†	0.0	153	151	+2.0	−0.50	+0.21	+0.30
E. L.	160	153	+7.0	151	157§	−6.0	152	152	0.0	+1.49	−0.64	−0.12
D. H.	160	157	+3.0	163	167§	−4.0	Still—A-V at 25 min.			+0.11	−0.93	−1.3
Mean	148.4		+0.5	147.7		+1.0	146.2		+1.0	−0.12	+0.07	−0.43
S. D.	±9.94			±9 7			±10.4			±0.79	±0.84	±1.3
S. E.	±2.57			±2.51			± 2.9			±0.20	±0.21	±0.35
t											0.35	1.23
p											>0.70‖	>0.20‖

* Fourteen to 17 minutes.
† Four minutes.
‡ Two minutes.
§ Seven minutes.
‖ Change from control value.

TABLE V

Myocardial arteriovenous differences of calcium before and after acetyl strophanthidin

	Calcium (*mEq./L.*)									Planimetered area in sq. in.		
	Control			Maximum*			Recovery†					
Patient	B.A.	C.S.	Diff.	B.A.	C.S.	Diff.	B.A.	C.S.	Diff.	I	II	III
M. P.	4.51	4.44	+0.07	4.57	4.18	+0.39	4.13	4.17	−0.04	+0.22	+0.51	+0.26
D. M.	4.39	4.46	−0.07	4.42	4.10	+0.32	4.17	4.17	0.0	−0.21	+0.30	−0.14
J. S.	4.12	4.05	+0.07	4.12	4.03	+0.09				−0.26	+0.09	
J. T.	4.33	4.42	−0.09	4.28	4.61	−0.33	4.60	4.85	−0.25	−0.28	−1.11	−0.90
J. T.	4.30	4.25	+0.05	4.35	4.47	−0.12	4.16	4.07	+0.09	−0.32	−0.25	+0.10
E. L.	4.96	5.05	−0.09	4.97	4.73	+0.24	5.03	4.88	+0.15	−0.50	+0.53	+0.33
E. R.	4.71	4.66	+0.05	4.65	4.87	−0.27	5.00	4.78	+0.22	+0.10	−0.52	+0.20
Mean	4.47		−0.01	4.48		+0.05	4.51		+0.02	−0.18	+0.12	−0.03
S. D.											±0.52	
S. E.											±0.20	
p											>0.20	

* Values at 4 to 7 minutes.
† Values at 14 to 17 minutes.

in Figure 1, shows a substantial negative phase of potassium transfer with the planimetered area of −0.94 square inch, representing a change of real significance (p less than 0.001). The subsequent period revealed a persistence of this negative phase through the 25 minute point that was probably significant. The actual cellular loss of potassium during Period II is not subject here to exact calculation, but, assuming a plasma flow of 50 ml. per 100 Gm. per minute and a cellular potassium content of the heart in failure at 6.3 mEq. per 100 Gm. (20), the decrement would range from 3 up to 7 per cent. There was no correlation between the potassium loss and the hemodynamic response to the drug ($r = 0.41$). In addition, there was no evident contrast in ionic change between those with and those without significant slowing of heart rate, in agreement with studies in the dog, in which prevention of rate changes failed to modify the strophanthidin potassium effect (21).

The simultaneous early rise in arterial potassium was too high to be derived solely from cardiac tissue, and probably represents mobilization from skeletal muscle (22). In two additional patients, sampling of the hepatic vein after strophanthidin revealed a slightly positive A-V difference of potassium, so that the liver is unlikely as a source area.

The simultaneous sodium determinations showed considerable variability in response, and the slight change in mean values for the group were of no statistical importance. In Table V, the calcium data on seven patients treated with strophanthidin are enumerated. The mean arterial levels were not significantly altered, nor were the planimetered areas for periods of the drug's activity significantly different from the control. The possibility remains of a larger group showing significant uptake of calcium, since this was the direction of ion movement in five of the seven patients.

DISCUSSION

The question of dosage and duration of administration must inevitably be considered in trying to assess the approximation of a digitalis preparation to toxicity. While a fixed dosage-electrocardiographic response may be predictable in normal animals, this, as noted by Friedberg (23), is often not the case in the clinical situation. In view of the fact that ventricular premature beats in a brief episode were seen in but 1 of the total of 23 patients after 1.1 mg. of strophanthidin, and that, in general, there was no specific digitalis effect on the electrocardiogram, this arbitrarily chosen dosage could be fairly adjudged as nontoxic. However, one cannot be certain that effects induced at a rapid rate of administration necessarily occur at slower rates. Conn's finding (24) of a qualitatively similar ionic alteration with chronic administration of digitoxin indicates that such a change is not limited to the particular circumstances of this experiment. It is presumed, then, that this telescoping of the events which usually

occur with digitalization enable the detection of changes in plasma concentration arising out of myocardial processes. The failure to demonstrate myocardial ionic exchange after cedilanid (25) can reasonably be ascribed to a less rapid mobilization of potassium.

The nonfeasibility of acquiring data in a given patient at progressive dosage levels makes reliance on animal studies necessary for the correlation of potassium change and hemodynamic activity over a range of dosage values. Vick and Kahn (26), using different concentrations of ouabain, were able to detect potassium losses whenever a positive inotropic effect was produced in the isolated guinea pig heart, losses which were small until excess of 50 per cent positive inotropic effect was produced. It is of interest that in two patients not included in this report 0.55 mg. of acetyl strophanthidin, one-half of the dosage chosen for this study, induced no detectable potassium change. Another patient receiving 0.9 mg. showed a potassium loss quite comparable to that following 1.1 mg.

The significance of the reduction in potassium content of the human heart in failure, as determined by the postmortem tissue analysis of Harrison, Pilcher and Ewing (22), is not clear, especially since other body tissues also participate in the negative potassium balance. It remains to be shown whether this altered potassium level influences contractility of the failing heart. The fact that the initial improvement in cardiac performance is associated with substantial egress of this ion, raises the question whether the maintenance of this new functional status is accompanied by a new equilibrium state with a diminished potassium gradient. The persistently negative phase of potassium net balance, after the peak therapeutic response has been attained, would indicate so. Even though the total exchangeable potassium content of the body tended to recover toward normal in the isotopic study of Aikawa and Fitz (27), the lowered myocardial content in autopsy specimens of digitalized patients (20) supports the above view.

Although the experiments with strophanthidin in the dog indicated that ventricular arrhythmias were associated with larger potassium outflow (7), there appeared in this study to be no difference based upon therapeutic response. Whereas the human heart undergoing a positive inotropic response always exhibited a potassium loss, the degree of change in mechanical function was not quantitatively related. A more direct measure of cardiac contractility than cardiac stroke work might lend added assurance to this conclusion. Moreover, even if the potassium changes were related to the functional response, the state of the basic contractile unit would be of prime importance amongst the determinants of cardiac response. Studies of a canine failing heart preparation have indicated substantial deviations from normal in the protein complex (1, 28). It would seem highly probable that in these patients there was variation in the myocardial protein of unknown extent, perhaps dependent upon the degree of destruction, dilatation, hypertrophy and cardiac decompensation among other factors.

Another interesting negative correlation was the general lack of characteristic repolarization changes on the precordial electrocardiogram despite the presence of substantial ionic shifts. As this manner of recording the process of electrical recovery correlates well with the direct cellular recording microelectrode (29), the manifestation of an altered recovery phase may require a more sustained ion effect.

The maintenance of a net loss of potassium despite the abruptly elevated arterial levels points to a potent effect on potassium transfer out of the heart. Whether inward flux is modified in any way by strophanthidin directly, or by the increased perfusing concentrations under the conditions of this experiment, is not known. But it is of note that this phenomenon did not appear to inhibit the positive inotropic response. Although the rise was a limited one, this finding would tend to support work on isolated heart (30), in which increasing concentrations of potassium in the perfusate were found to have no effect on the therapeutic actions of ouabain despite an effective antagonism in the presence of toxic dosages.

Despite the fact that a unitary theory of myocardial contraction based on ionic changes is not justified, it is worth noting that both digitalis and veratrum (26) preparations precipitate potassium egress from the heart simultaneous with the positive inotropic effect. These instances, at least, fulfill the requirements of the reduced internal ionic content theory (4). The converse ionic movement found with adrenalin (31) and noradrenalin (32) would appear to rule out a uni-

versal application of the simple relationship postulated by this theory. Since Gruhzit and Farah (33) were able to block the glycoside inotropic effect with dinitrophenol, but not the inotropic effect of adrenalin, separate mechanisms of action for these agents are probable. Nevertheless, the obligatory participation of the ionic transfers in their therapeutic effect is unproven.

Although no simultaneous movement of sodium was found in these patients, the fact that a small change might be obscured by the high plasma levels of this ion is an important consideration, and would make cellular analysis desirable to settle the question whether electroneutrality is maintained by an equivalent exchange of monovalent cation. Failure to find such evidence would lead to a search for anion moving with potassium; one intriguing possibility is suggested by the finding of amino acid reduction in digitalis induced contracture (34).

The failure, in the seven patients studied, to find evidence that the transfer of calcium ion in the heart is altered in a consistent fashion during the course of strophanthidin's activity does not necessarily detract from Loewi's concept of the role of extracellular calcium acting on the cell membrane to facilitate contraction in this circumstance (35), providing one assumes that the calcium, so acting, does not alter the concentration equilibrium between interstitial fluid and capillary. If its specific effect is to modify permeability to potassium, the perfusion concentration of calcium might be expected to relate quantitatively to the egress of potassium. Within limits of the levels observed in these patients, no such parallelism was found. Further clarification of the status of net calcium transfer in this situation may be attainable through measuring arteriovenous concentration differences of ultrafiltrable calcium. Whereas large changes in this biologically active moiety should be reflected in assays of total concentration, a minute change may remain undetected.

The finding of a significantly increased concentration of arterial lactate and pyruvate suggests a reduced ability of some tissues, at least, to store or utilize these substances for energy production in the course of untreated cardiac failure. The extraction of glucose, lactate and pyruvate by the heart was maintained normally although at a higher perfusion level. Strophanthidin appears to modify carbohydrate metabolism of extracellular tissue, whether or not increased peripheral blood flow occurs, since an appreciable fall in arterial lactate and pyruvate took place. While the myocardial extraction of both was normal at 7 minutes after infusion, that of lactate was reduced at 15 minutes to a probably significant degree ($p < 0.05 > 0.02$), and may indicate among other alternatives an accelerated production by glycolytic pathways during this time. A clarification of this interpretation from various experiments on isolated tissue is not obtainable, particularly since, as Olson has indicated (1), the conditions of these studies, notably the stimulatory effect upon tissue respiration, preclude comparison with *in vivo* experiments.

The association of potassium loss and glycogenolysis in liver metabolism, the latter process reaching a peak after the ionic change in epinephrine studies (36), bears a resemblance to these findings with strophanthidin. Although the potassium movement has been dissociated from the hepatic glycogenolytic process through the use of adrenergic blockade (37), their mutual independence in the heart has not been established.

SUMMARY

Preliminary to an examination of the myocardial metabolic changes induced by acetyl strophanthidin, the hemodynamic effect of 1.1 mg. intravenously was studied in five patients with low output heart failure. A substantial stroke work increase occurred within five minutes, becoming maximal at nearly twice the control level by 15 minutes and declining in 25 minutes.

The same rapid mode of administration was employed in 18 patients with cardiac decompensation in whom myocardial extractions of electrolytes and carbohydrate substrate were determined by paired arterial and coronary sinus sampling. The significantly greater increment in coronary sinus potassium, usually maximal within four minutes, indicated a net myocardial loss of this ion which was not counteracted by a directional reversal of this ionic movement during the observation period. This phenomenon was not quantitatively related to the stroke work response and was not, with one exception, associated with toxic electrocardiographic manifestations. Likewise, with few ex-

ceptions, evidence of an altered repolarization phase on the precordial electrocardiogram was lacking. No significant changes were observed in the extractions of sodium and calcium.

Despite elevated arterial concentrations of glucose, lactate and pyruvate, the myocardial extraction of these substances was within normal limits prior to therapy. Following strophanthidin the arterial levels of lactate and pyruvate diminished, a change which was apparently not dependent on total blood flow increase. Extractions were not significantly altered until 15 minutes, when a reduction in the arteriovenous difference of lactate was noted. The possible relationship of this finding to potassium egress from the heart was considered.

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ANNOUNCEMENTS OF MEETINGS

The Fifty-First Annual Meeting of THE AMERICAN SOCIETY FOR CLINICAL INVESTIGATION will be held in Atlantic City, N. J., on Monday, May 4, 1959, at 9:00 A.M. at the Casino Theater on the Steel Pier.

The Sixteenth Annual Meeting of THE AMERICAN FEDERATION FOR CLINICAL RESEARCH will be held in Atlantic City, N. J., at the Casino Theater on the Steel Pier on Sunday, May 3, 1959, at 9:00 A.M.

THE ASSOCIATION OF AMERICAN PHYSICIANS will hold its Seventy-Second Annual Meeting in Altantic City, N. J., at the Casino Theater on the Steel Pier on Tuesday, May 5, 1959, at 9:30 A.M. and in the Vernon Room, Chalfonte-Haddon Hall, on Wednesday, May 6, 1959, at 9:30 A.M.

STUDIES ON THE RELATIONSHIP BETWEEN GLUCOSE OXIDATION AND INTERMEDIARY METABOLISM. III. THE INFLUENCE OF PYRIDINE NUCLEOTIDES ON PROTEIN SYNTHESIS * †

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There is now abundant evidence that in many animal tissues glucose can be oxidized by two biochemical routes—the Embden-Meyerhof pathway and the hexosemonophosphate shunt. A number of investigators have described the enzymatic steps (3, 4) as well as the quantitative significance (5) of these two routes of glycolysis. Studies in this laboratory have recently been concerned with the role of glycolysis, and particularly of the hexosemonophosphate shunt, in regulating the various synthetic processes of the animal cell. Previous reports have dealt with the relationship between glycolysis and fatty acid and cholesterol synthesis (6–8).

Glucose oxidation is also known to have a profound effect on protein metabolism. While marked restriction of caloric intake both in man and animals results in accelerated protein wastage, the administration of carbohydrate alone to such subjects causes a marked diminution of this nitrogen loss. This capacity of carbohydrate to spare protein cannot be a consequence simply of added calories; the amount of carbohydrate required is trivial, and, moreover, similar amounts of fat are relatively ineffectual in this regard (9). It is obvious, therefore, that carbohydrate and protein metabolism are intimately interlinked; yet the mechanism whereby this articulation is accomplished remains obscure.

We have previously postulated on theoretical grounds that this influence of carbohydrate on protein metabolism is mediated by the cofactors generated by the hexosemonophosphate shunt (10), and evidence has recently been presented that these cofactors may in fact play a role in the regulation of protein synthesis (1, 2). The purpose of the present paper is to present in detail these studies on a possible mechanism for the protein-sparing effect of glucose.

EXPERIMENTAL PROCEDURE

Treatment of animals. Male rats of the Long-Evans strain, weighing from 150 to 250 Gm., were used throughout this study. The animals were fed three different diets. One group was allowed free access to Purina Laboratory Chow prior to the time of death. Two other groups were placed in restraining cages and given *ad libitum* a 25 per cent protein diet [1] or a protein-free diet [1] for 48 to 72 hours prior to death. These variations in diet were not found to produce differences in the rates of protein synthesis by rat liver homogenates in the experiments reported here.

Preparation of homogenates and incubation procedure. The rats were stunned by a blow on the head and exsanguinated by cutting their throats. The livers were perfused through the portal vein with 15 ml. of an ice cold sucrose-electrolyte buffer (pH 7.8) described by Zamecnik and Keller (11). The livers were then excised, placed in cold buffer, and allowed to cool; they were weighed and transferred to a Dounce homogenizer to which was added a volume of sucrose-electrolyte buffer equal to the weight of the liver. The livers were then homogenized using four slow strokes with each of two plungers, the tightest of which had a clearance of 1 mm.

[1] The composition of the 25 per cent protein diet was as follows: glucose, 67 per cent; casein, 25 per cent; Hawk-Oser salt mixture, 7 per cent; and vitamins per 1,000 Gm. of diet—inositol, 500 mg.; folic acid, 10 mg.; 0.1 ml. oleum percomorphum containing vitamin A, 6,000 units; vitamin D, 850 units; and one bottle of Folbesyn® (Lederle) containing thiamine, 10 mg.; riboflavin, 10 mg.; sodium pantothenate, 10 mg.; niacinamide, 50 mg.; pyridoxine, 5 mg.; B-12, 15 μg.; and ascorbic acid, 300 mg. The composition of the protein-free diet was the same as the 25 per cent protein diet except that it contained: glucose, 80 per cent; corn oil, 15 per cent; Hawk-Oser salt mixture, 5 per cent; and no casein.

* This work was presented at the meeting of the Society for Clinical Investigation, May 5, 1958, and has been published in abstract form (1, 2).

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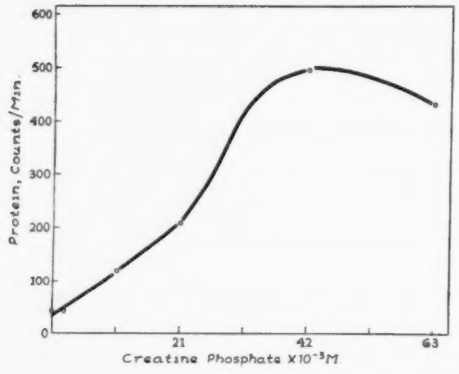

FIG. 1. INFLUENCE OF ATP-GENERATING SYSTEM ON PROTEIN SYNTHESIS

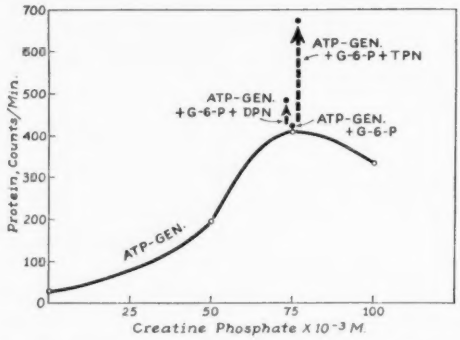

FIG. 2. INFLUENCE OF TPN AND DPN ON PROTEIN SYNTHESIS

The entire procedure was carried out in a room maintained at 5° C. The resulting homogenate was centrifuged at 800 × G for 10 minutes at −1° C. to remove intact cells and nuclei, and the supernatant, containing the remainder of the cellular components, was used in these experiments.

One ml. aliquots of the homogenate were placed in centrifuge tubes, and substrate, coenzymes, water and acetate-1-C^{14} or DL-valine-1-C^{14} were added to give a constant volume. The flasks were gassed with 95 per cent oxygen-5 per cent carbon dioxide for 10 seconds, sealed with serum caps, and incubated at a 30° angle in a Dubnoff metabolic shaker for 45 minutes at 37.5° C.

Analytical methods. Protein was purified by a modification of the method described by Littlefield, Keller, Gross and Zamecnik (12). At the end of the incubation the serum caps were removed. The protein was precipitated, centrifuged and resuspended three times with 5 ml. of 10 per cent trichloracetic acid. The protein-trichloracetic acid mixture was then heated to 90° C. in a water bath for 15 minutes (13), following which it was cooled, centrifuged and decanted. The precipitate was then washed two times with 8 ml. alcohol-ether-carbon tetrachloride (2:2:1) by shaking the capped centrifuge tubes for five minutes on an International Bottle Shaking Machine. The suspension was again centrifuged and the supernatant decanted. Five ml. 1 N NaOH was then added to the precipitate, and the opalescent solution was warmed to 40° C. for 30 minutes (14). The protein was then reprecipitated by the addition of 2 ml. 10 N HCl; the tubes were centrifuged and the supernatant was decanted. The precipitate was then washed once with 5 ml. of 10 per cent trichloracetic acid and twice with 5 ml. of acetone. In these experiments the initial addition of carrier acetate and valine and the use of hot alcohol-ether-carbon tetrachloride were found to have no effect on the final result.

The acetone-washed precipitates (weighing approximately 10 mg.) were then dissolved in 1 ml. Hyamine® [2] hydroxide as described by Vaughan, Steinberg and Logan (15). The Hyamine® protein solution was transferred into glass vials to which was added 16 ml. of 0.4 per cent diphenyloxazole in toluene, and the radioactivity was assayed in a Packard Liquid Scintillation Counter which was set at 1,120 volts in order to minimize the quenching of counts.

To determine the location of the label in the protein end-product, N-terminal amino acids were separated from the protein as their dinitrophenyl derivatives as described by Porter (16). The protein was suspended in 1 ml. 5 per cent $NaHCO_3$. Two ml. of 10 per cent fluorodinitrobenzene in ethanol was added, and the mixture was shaken on an International Bottle Shaking Machine for two hours. This solution was centrifuged, and the precipitate was washed two times each with water, ethanol, and ether and air-dried in a vacuum oven. The dinitrophenyl-protein was then hydrolyzed in 5.7 N HCl by heating in an autoclave at 120° C. and 15 pounds pressure for four hours. The protein hydrolysate was extracted three times with ethyl ether. The ethyl ether extracts were combined, taken to dryness, and dissolved in a few drops of ethanol. Aliquots of the ether extract and the protein hydrolysate were then mounted at "zero mass" on stainless steel planchettes and assayed for radioactivity in a Nuclear Chicago flow counter equipped with a micromil window.

RESULTS

Influence of pyridine nucleotides on protein synthesis from acetate

The results of studies on the effects of pyridine nucleotides on protein synthesis are shown in

[2] Hyamine® 10X is the trade name for para diisobutyl cresoxy ethoxy ethyl dimethyl benzyl ammonium chloride monohydrate which was purchased from The Rohm and Haas Company.

TABLE I

The influence of pyridine nucleotides on protein synthesis from acetate-1-C^{14} in rat liver homogenates *

Additional substrate and/or cofactors	Acetate-1-C^{14} converted to protein (*cpm*)				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5†
None	180	50	25	30	90
G-6-P	123	106	33	23	71
G-6-P + TPN	665	141	147	95	335
G-6-P + DPN	612	101	83	56	243
G-6-P + TPN + DPN	506	183		93	242

* Rats in Experiment 1 fed Purina Laboratory Chow; rats in Experiments 2 through 5 fed 25 per cent protein diets. For details see text.

Concentration of additions:

Experiments 1 and 5: G-6-P, 12×10^{-3} M; DPN and TPN, 0.5×10^{-3} M; creatine phosphate, 85×10^{-3} M; ATP, 1.7×10^{-3} M + 2 μc. acetate-1-C^{14} to make a final volume of 2.1 ml.

Experiment 2: G-6-P, 14×10^{-3} M; DPN and TPN, 0.5×10^{-3} M; creatine phosphate, 97×10^{-3} M; potassium acetate, 2.7×10^{-3} M; ATP, 1.9×10^{-3} M + 2 μc. acetate-1-C^{14} to make a final volume of 1.8 ml.

Experiments 3 and 4: G-6-P, 8×10^{-3} M; DPN and TPN, 0.6×10^{-3} M; creatine phosphate, 112×10^{-3} M; potassium acetate, 3.1×10^{-3} M; ATP, 2.2×10^{-3} M + 2 μc. acetate-1-C^{14} to make a final volume of 1.6 ml.

† Alloxan-diabetic rats.

Table I. Glucose-6-phosphate served as the substrate in these experiments, and as indicated in Table I the addition of this compound alone had no stimulatory effect on protein synthesis in four out of the five experiments. On the other hand, the addition of either diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) in every case caused a definite stimulation of protein synthesis. It should be noted that in each of the five experiments the addition of TPN produced a greater increase in the synthesis of protein than did DPN. These effects were not additive in that a combination of TPN and DPN did not significantly enhance protein synthesis above that obtained with TPN alone. In a single experiment protein synthesis in homogenates obtained from the livers of alloxan diabetic rats was studied; the results were identical to those obtained in the experiments on normal rats.

The influence of other glycolytic cofactors upon protein synthesis from acetate

A previous study has demonstrated that glucose oxidation may be selectively stimulated down either the hexosemonophosphate shunt or the Embden-Meyerhof pathway by the addition of TPN or DPN, respectively (17). Thus, it was possible that the effects of the pyridine nucleotides on protein synthesis observed here might simply be the passive consequence of the enhanced production of either adenosine triphosphate (ATP) or guanosine triphosphate (GTP), two cofactors previously shown to stimulate protein synthesis (11, 18).

To resolve this question the effects of ATP and GTP, both alone and in the presence of added pyridine nucleotide, were examined. Figure 1 shows the influence of an ATP-generating system, creatine phosphate plus ATP, on protein synthesis in the rat liver system studied here. As has been previously noted by Zamecnik and Keller (11), increasing concentrations of creatine phosphate produces an increasing stimulation of protein synthesis. This effect is seen to reach a plateau in the experiment shown in Figure 1 at approximately a 40 to 45 μM concentration of creatine phosphate, after which there is a slight decrease in the rate of protein synthesis with increasing concentrations of creatine phosphate.

It is apparent therefore that the influence of the pyridine nucleotides upon protein synthesis could simply be due to increased ATP generation. In order to establish whether the stimulation of protein synthesis by TPN and DPN is in fact independent of ATP generation, the influence of the pyridine nucleotides on protein synthesis was determined during the *maximal* enhancement of protein synthesis by ATP. As is illustrated in Figure 2, if G-6-P and TPN were added to a homogenate at the point on the curve of creatine phosphate stimulation at which protein synthesis was maximal, a twofold increase in protein synthesis was produced. G-6-P plus DPN produced

TABLE II

The influence of pyridine nucleotides on protein synthesis from acetate–1–C^{14} in the presence of maximal ATP-generation in liver homogenates *

Additional substrate and/or cofactors	Acetate–1–C^{14} converted to protein (*cpm*)																			Average increase produced by cofactors (*cpm*)
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Exp. 7	Exp. 8	Exp. 9	Exp. 10	Exp. 11	Exp. 12	Exp. 13	Exp. 14	Exp. 15	Exp. 16	Exp. 17	Exp. 18	Exp. 19	Exp. 20	
None	180	50	30	22	25	35	1	111	133	74	142	128	151	90	140	258	41	57	78	52	
G-6-P + $CrPO_4$	694	181	156	300	422	377	117	122	182	142	250	137	178	292	110	340	131	161	151	114	+138
G-6-P + $CrPO_4$ + TPN	988	336	334	665	668	1,073	431	298	353	244	538	337	403	363	310	676	439	531	227	221	+382
G-6-P + $CrPO_4$ + DPN	920	314	236	474	479	573	701	315	343	232	454	199	402	254	166	689	377	482	163	229	+310
G-6-P + $CrPO_4$ + TPN + DPN	796	261	299	575	596	817	372	284	345	205	474	407	606	314	223	493	416	516	230	209	+332

* Animals used in Experiments 1, 2, 10, 17 and 18 were fed Purina Laboratory Chow; rats in the remainder of the experiments were fed 25 per cent protein diets. For details see text.

Concentration of additions:

Experiment 1: G-6-P, 12×10^{-3} M; creatine phosphate, 85×10^{-3} M; ATP, 1.7×10^{-3} M; DPN and TPN, 0.5×10^{-3} M + 2 μc. acetate–1–C^{14} to make a final volume of 2.1 ml.

Experiment 2: G-6-P, 14×10^{-3} M; creatine phosphate, 93×10^{-3} M; ATP, 1.9×10^{-3} M; DPN and TPN, 0.5×10^{-3} M; potassium acetate, 2.7×10^{-3} M + 2 μc. acetate–1–C^{14} to make a final volume of 1.8 ml.

Experiments 3 through 5: G-6-P, 16×10^{-3} M; creatine phosphate, 112×10^{-3} M; ATP, 2.2×10^{-3} M; DPN and TPN, 0.6×10^{-3} M; potassium acetate, 3.1×10^{-3} M + 2 μc. acetate–1–C^{14} to make a final volume of 1.8 ml.

Experiments 6 through 20: G-6-P, 8×10^{-3} M; creatine phosphate, 112×10^{-3} M; ATP, 2.2×10^{-3} M; DPN and TPN, 0.6×10^{-3} M; potassium acetate, 3.1×10^{-3} M + 2 μc. acetate–1–C^{14} to make a final volume of 1.8 ml.

a lesser increase in protein synthesis while G-6-P alone caused no effect.

In Table II are listed the results of 20 similar experiments in which TPN and DPN were added to systems in which ATP generation was maximal. In each of these experiments the addition of the pyridine nucleotides to the systems containing glucose-6-phosphate and creatine phosphate resulted in a further increase in protein synthesis. Moreover, in 10 of the experiments the effect of TPN was definitely greater than that of DPN. In only 1 experiment was the effect of DPN greater, and in the remaining 9 experiments the stimulatory effects of TPN and DPN were comparable. In all but one experiment the effects of TPN and DPN were not additive.

To determine whether the influence of pyridine nucleotides on protein synthesis was also independent of enhanced GTP generation, another series of experiments was performed (Figure 3). Increasing concentrations of GTP were added to a system in which creatine phosphate concentration was maximal. As in the case of ATP, at near optimal levels of GTP, TPN and DPN enhanced protein synthesis still further. The results of four such experiments are given in Table III. GTP consistently enhanced protein synthesis above levels attainable with maximal ATP generation, and in each instance the addition of pyridine nucleotide produced still greater protein synthesis than was attainable with GTP. In two of the experiments the effect of TPN was greater than that of DPN. In the other experiments TPN and DPN had similar effects.

FIG. 3. INFLUENCE OF GTP, TPN AND DPN ON PROTEIN SYNTHESIS

TABLE III

The influence of pyridine nucleotides on protein synthesis from acetate–1–C^{14} in the presence of GTP in rat liver homogenates *

Additional substrate and/or cofactors	Acetate–1–C^{14} converted to protein (*cpm*)			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
None	41	57	52	0
G–6–P + $CrPO_4$	157	161	114	
G–6–P + $CrPO_4$ + GTP	181	202	119	665
G–6–P + $CrPO_4$ + GTP + TPN	302	576	159	733
G–6–P + $CrPO_4$ + GTP + DPN	226	579	171	561
G–6–P + $CrPO_4$ + GTP + TPN + DPN	263	559	184	657

* Rats in Experiments 1 and 2 fed Purina Laboratory Chow; rats in Experiments 3 and 4 fed 25 per cent protein diet. For details see text.

Concentration of additions: creatine phosphate, 73×10^{-3} M; TPN and DPN, 0.61×10^{-3} M; GTP, 0.12×10^{-3} M; G–6–P, 15.2×10^{-3} M; ATP, 2.4×10^{-3} M; potassium acetate, 3.0×10^{-3} M + 2 μc, acetate–1–C^{14} to make a final volume of 1.65 ml.

Effect of pyridine nucleotides on protein synthesis from valine

Because of the possibility that the effects of the pyridine nucleotides on acetate-1-C^{14} incorporation into protein might not necessarily represent enhanced protein synthesis *per se* but rather reflect an increased incorporation of the labeled acetate into four carbon amino acid precursors, it was necessary to study the effects of TPN and DPN on the incorporation of an *essential* amino acid into protein. In Table IV are given the results of five experiments in which the effects of TPN and DPN on the incorporation of DL-valine-1-C^{14} into protein were studied. As in the case of acetate, creatine phosphate and glucose-6-phosphate produced a definite increase in protein synthesis when added to the liver system. Once again, DPN and TPN produced a further increase in protein synthesis. In two instances TPN had a greater effect than DPN; in the remaining three experiments there was no apparent difference between the stimulatory effects of the two pyridine nucleotides.

Site of label in synthesized protein

Since evidence has recently been presented that incorporation of labeled amino acids into protein may under some circumstances represent acylation by the amino acids rather than their actual incorporation into the peptide chain itself (19), the site of the label in proteins synthesized by the system employed here was examined.

Regenerating livers from partially hepatectomized rats were studied in order to obtain a highly active preparation. Homogenates of these livers were incubated with valine-1-C^{14} and with the various combinations of cofactors described above. The isolated proteins incorporated the C^{14} labeled valine as shown in the second column of Table V.

TABLE IV

Influence of pyridine nucleotides on protein synthesis from DL–valine–1–C^{14} in rat liver homogenates *

Additional substrate and/or cofactors	DL–valine–1–C^{14} converted to protein (*cpm*)				
	Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5
None	68	16	140	9	0
G–6–P + $CrPO_4$	249	282	364	112	20
G–6–P + $CrPO_4$ + TPN	269	386	390	202	170
G–6–P + $CrPO_4$ + DPN	298	331	377	223	111
G–6–P + $CrPO_4$ + TPN + DPN	127	292	399	171	164

* Rats in Experiments 1 and 2 fed 25 per cent protein diets; rats in Experiments 3 through 5 fed protein-free diets. For details see text.

Concentration of additions:

Experiment 1: G–6–P, 16×10^{-3} M; DPN and TPN, 0.6×10^{-3} M; creatine phosphate, 112×10^{-3} M; DL–valine, 3.1×10^{-3} M; ATP, 2.2×10^{-3} M + 2 μc. DL–valine–1–C^{14} to make a final volume of 1.6 ml.

Experiments 2 through 5: G–6–P, 8×10^{-3} M; DPN and TPN, 0.6×10^{-3} M; creatine phosphate, 112×10^{-3} M; DL–valine, 3.1×10^{-3} M; ATP, 2.2×10^{-3} M + 2 μc. DL–valine–1–C^{14} to make a final volume of 1.6 ml.

TABLE V

Distribution of C^{14} in protein synthesized from DL-valine-1-C^{14} in rat liver homogenates *

Additional substrate and/or cofactors	DL-valine-1-C^{14} converted to protein (*cpm*)	Per cent recovered counts in: Fluorodinitrobenzene reacting amino acids	Per cent recovered counts in: Protein residue
None	161	3	97
G-6-P + $CrPO_4$	1,756	1	99
G-6-P + $CrPO_4$ + TPN	1,925	1	99
G-6-P + $CrPO_4$ + DPN	2,197	0	100
G-6-P + $CrPO_4$ + TPN + DPN	1,955	1	99

* Partial hepatectomy performed four days prior to death. Rats fed Purina Laboratory Chow.
Concentration of additions: ATP, 2.7×10^{-3} M; G-6-P, 8.3×10^{-3} M; creatine phosphate, 53×10^{-3} M; DPN and TPN, 0.66×10^{-3} M + 2 μc. DL-valine-1-C^{14} to make a final volume of 1.5 ml.

The dinitrophenyl derivatives of the N-terminal amino acids were formed and isolated. The radioactivity in this fraction was in each case negligible compared to that of the intact protein, a finding which strongly suggests that under the conditions of synthesis and isolation employed in this study the valine-C^{14} was in fact incorporated into the peptide linkages of the protein.

DISCUSSION

Evidence has recently been presented that the oxidation of glucose exerts a controlling influence on both fatty acid and cholesterol synthesis, not so much by producing substrate but rather by generating the reduced pyridine nucleotides, particularly triphosphopyridine nucleotide, required for these synthetic reactions (6–8). It seemed possible, therefore, that the interrelation of carbohydrate and protein metabolism might be disclosed by an examination of the relationship between protein synthesis and the cofactors generated during glucose breakdown.

In Figure 4 are shown the cofactors produced by the oxidation of glucose to pyruvate and oxalacetate. As glucose enters the cell it may be metabolized by one of two pathways. If it goes down the Embden-Meyerhof route, reduced diphosphopyridine nucleotide (DPNH) is generated, whereas oxidation by the hexosemonophosphate shunt yields reduced triphosphopyridine nucleotide (TPNH). Furthermore, glycolysis can also produce adenosine triphosphate (ATP), and if oxalacetate is formed the synthesis of guanosine triphosphate (GTP) results.

The studies reported here clearly indicate that each of the cofactors involved in glycolysis and oxalacetate formation, GTP, ATP, DPN and TPN, can exert a stimulatory effect on protein synthesis, as judged by their influence on the incorporation of acetate and valine into the protein of rat liver homogenates.

ATP and GTP have previously been shown to enhance protein synthesis in liver homogenates (11, 18). The most significant finding of the present study, however, was the observation that the pyridine nucleotides, DPN and TPN, probably in their reduced forms, consistently produce a stimulation of protein synthesis. This effect was particularly apparent in the case of TPN. Furthermore, the demonstration that the influence of TPN and DPN on protein synthesis is very marked even in the presence of stimulation by ATP and GTP indicates that the effects of the pyridine nucleotides on protein synthesis are independent of the role they may play in increasing ATP or GTP generation via glycolysis or the Krebs cycle.

In order to examine the mechanisms by which the various cofactors produced during glycolysis may exert their influence on protein synthesis, the known biochemical steps from the Krebs cycle intermediate, α-ketoglutarate, to the synthesis of proteins are summarized in Figure 5. The first of these reactions consists of the fixation of ammonia with α-ketoglutaric acid to form glutamic acid. The enzyme responsible for this reaction, glutamic dehydrogenase, is known to require either DPNH or TPNH as cofactor (20). Second, glutamate can yield other nonessential amino acids by transamination reactions. Third, glutamic acid or its

transamination products are activated in the presence of ATP to form the adenylate derivatives of the amino acids (21). Finally, these activated amino acids are incorporated into the peptide chains of protein in a series of steps requiring ribonucleic acid and GTP (18).

It is apparent, therefore, that of the four cofactors produced during glycolysis—TPNH, DPNH, ATP and GTP—each is *in fact* known to be involved in the conversion of α-ketoglutarate to proteins. That each of these cofactors may be rate-limiting for the synthesis of proteins in liver homogenates has been demonstrated by the data presented here. It is of particular interest that reduced pyridine nucleotides can exert a controlling influence upon protein synthesis since, as shown above, these cofactors are required for ammonia fixation with α-ketoglutaric acid, the only reaction in mammalian tissues by which a ***net*** synthesis of amino acids can take place. TPNH is also involved in the fixation of CO_2 to form the precursors of α-ketoglutarate. These findings would imply that the rate of formation of amino acids themselves may be rate-limiting in the synthesis of protein.

Furthermore, it should be noted that while both TPN and DPN exerted a direct stimulatory effect on protein synthesis, TPN frequently was the more effective cofactor in this regard. This finding suggests that TPN (in the form of TPNH) may play a greater role in the regulation of protein synthesis than does DPNH. Further evidence to support this view is provided by the fact that while glutamic dehydrogenase can utilize either TPNH or DPNH in glutamic acid synthesis (20), the level of DPNH in the mitochondria of the liver cell is probably too low to enable this cofactor to be an important source of hydrogen ions in the synthesis of glutamic acid (22).

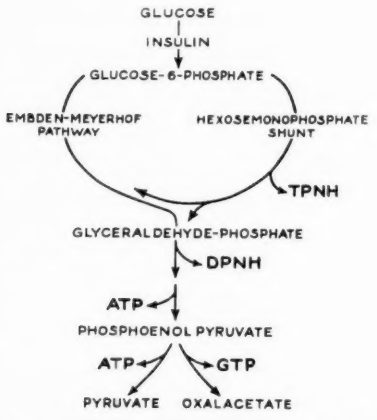

FIG. 4. COFACTORS PRODUCED DURING GLYCOLYSIS

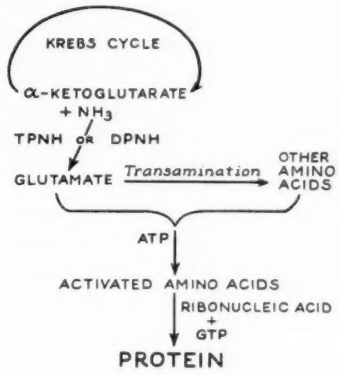

FIG. 5. COFACTORS INVOLVED IN PROTEIN SYNTHESIS

The demonstration that the pyridine nucleotides can be a limiting factor in the synthesis of proteins in the system described here raises the question of the relationship between this *in vitro* effect and the protein-sparing effect of glucose observed in intact animals. While the obvious limitations of using the results of *in vitro* experiments to explain *in vivo* phenomena clearly apply to this problem, there is evidence that in the intact liver an increase in protein synthesis does accompany the administration of glucose (23). It seems justifiable, therefore, to hypothesize from the findings reported here that the protein-sparing effect of glucose is partially due to an increase in synthesis mediated by the cofactors generated during glycolysis and especially by the pyridine nucleotides.[3] Furthermore, the fact that TPNH is more frequently the limiting factor in protein synthesis in cell-free systems suggests that the generation of this cofactor and hence the glucose oxidized via the hexosemonophosphate pathway may be of particular importance in the sparing of protein by glucose.

[3] It should be pointed out in this connection that the phenomena observed here are not limited to hepatic tissue in that the pyridine nucleotides have also been found to be rate-limiting in the synthesis of proteins in the regenerating dog kidney (24).

SUMMARY AND CONCLUSIONS

The relationship between glycolysis and protein synthesis has been examined utilizing a cell-free system of rat liver. It was found that each of the cofactors produced by glucose oxidation can exert a stimulatory effect on the synthesis of protein, as measured by the rates of incorporation of acetate-1-C^{14} or valine-1-C^{14}. Of particular note was the finding that the pyridine nucleotides, and especially triphosphopyridine nucleotide, will consistently enhance protein synthesis; furthermore, this effect appears to be independent of the influence of these coenzymes on the generation of adenosine triphosphate or guanosine triphosphate. The relationship between these observations and the protein-sparing effect of glucose is discussed.

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STUDIES ON THE MECHANISM OF THE LUPUS ERYTHEMATOSUS (L.E.) PHENOMENON *

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Since the original demonstration by Hargraves of the Feulgen positive nature of the lupus erythematosus (L.E.) cell (1), it has become increasingly evident that the specific nature of the L.E. phenomenon resides in the reaction of the cell nucleus with a gamma globulin-like protein from the L.E. patient's serum (2–8). Friou, Finch and Detre in particular (3, 4), and others (5, 6) have shown by means of the fluorescent antibody technique of Coons and Kaplan (9) that there is nuclear localization of serum gamma globulin of patients with L.E.

The present communication on the L.E. phenomenon is divided into two parts. The first part, utilizing L. E. tests done with isolated cell nuclei and purified L.E. gamma globulin, presents evidence for the participation of an accessory serum factor necessary for phagocytosis in the L.E. phenomenon. The second part contains quantitative studies with fluorescent-labeled L.E. gamma globulin which suggest that the L.E. factor is not identical in all patients, and that the L.E. factor may react with a protein of the cell nucleus.

METHODS AND MATERIALS

Preparation of gamma globulin and nuclei. Sera were obtained from normal controls and from clinically typical cases of L.E.D. (lupus erythematosus disseminata) with strongly positive L.E. tests diagnosed on the wards of the Massachusetts General Hospital. Gamma globulin was prepared from these sera by ethanol fractionation, employing a modification of Methods 6 and 10 of Cohn (10).

Nuclei from the livers of 200 to 300 Gm. male rats (Tables III through VI) or from human buffy coat (Tables I and II) were freshly prepared each day except where noted, by fractionation in 0.25 sucrose (11) following homogenization in a glass Potter-Elvejhem homogenizer. Following the customary wash in isotonic sucrose, the liver nuclei were washed two additional times in buffered $CaCl_2$ sucrose (42.8 Gm. sucrose, 0.37 Gm. $CaCl_2$, 4.0 Gm. NaCl, 0.69 Gm. $NaH_2PO_4 \cdot H_2O$ per L., adjusted to pH 7.0 with NaOH).

Studies utilizing the L.E. test. L.E. tests were performed by a modification of the procedure of Zinkham and Conley (12) which employs glass beads to traumatize the leukocytes. Buffy coat (0.5 ml.) was obtained from heparinized normal blood by the addition of bovine fibrinogen (13) and the pellet obtained by centrifugation was resuspended in 0.2 ml. of an isotonic NaCl solution of the gamma globulin to be tested, 0.05 ml. of 0.1 M pH 7.4 phosphate buffer, and 0.15 ml. of isotonic NaCl. After addition of two glass beads, the tubes were shaken at a rate of 240 oscillations per minute with an Eberbach shaking machine for 50 minutes at room temperature. At the end of this time the tubes were centrifuged, and the pellets smeared on cover slips, stained with Wright's stain, and the number of L.E. cells per 1,000 leukocytes was determined.

Experiments designed to demonstrate the necessity of an accessory serum factor for the L.E. phenomenon (Table I) were done in the following manner. Leukocytes from 0.5 ml. of normal buffy coat were washed five times with buffered saline (8 Gm. of NaCl and 1.38 Gm. of $NaH_2PO_4 \cdot H_2O$ per L. adjusted to pH 7.0 with NaOH) and incubated with 0.3 ml. of isotonic saline (first column of Table I), 0.2 ml. of L.E. gamma globulin heated to 56° C. for 30 minutes (second column), and with 0.2 ml. of heated L.E. gamma globulin with 0.1 ml. of fresh normal serum added (third column). The phagocytic index was determined with particulate starch (14) on separate tubes, by counting the number of granules per 100 white blood cells with the aid of a phase contrast microscope.

The experiments designed to demonstrate the two-stage nature of the L.E. phenomenon (Table II) were done in a somewhat different manner. Nuclei either from 1.0 ml. of fresh buffy coat, or 1.2 mg. of lyophilized leukemic nuclei, were incubated with 0.2 ml. of L.E. gamma globulin at room temperature for 30 minutes with occasional agitation. The nuclei were then washed twice with buffered saline to remove unbound L.E. gamma

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TABLE I

Studies on the effect of lupus gamma globulin, normal serum factor, desoxyribonuclease and $MgCl_2$ on L. E. cell formation

Expt.	Addition	Control		L. E. gamma globulin		L. E. gamma globulin + normal serum	
		L. E. cells	Phagocy.	L. E. cells	Phagocy.	L. E. cells	Phagocy.
1	None	0		0		327	
	0.005 M $MgCl_2$	0		78		1,020	
2	None	0		33		2,460	
3	None		0		8		1,420
4	0.005 M $MgCl_2$	0		440		4,140	
	0.005 M $MgCl_2$ + 0.1 mg. DNAse	0		0		0	

globulin, and then reincubated with leukocytes from 0.5 ml. of buffy coat suspended in 0.2 ml. of normal serum. At the end of the second 30 minute incubation the tubes were centrifuged, the sediment smeared and stained, and the number of L.E. cells counted. As controls for unbroken cells in the preparation of fresh nuclei, duplicate preparations were run and smeared and counted after the initial incubation.

Studies with fluorescent lupus gamma globulin. Gamma globulin was conjugated with fluorescein isocyanate by the method of Coons and Kaplan (9). Nonspecific fluorescence was removed by dialysis for 24 hours in the cold against buffered saline, and by one or two passages through Dowex-1 chloride (15). Liver powder was not used because it removed specific nuclear fluorescence. Following passage through Dowex, the conjugate was concentrated about threefold in a dialysis bag by evaporation in the cold, and then redialyzed against buffered saline. A normal gamma globulin preparation was conjugated with each L.E. globulin. Conjugates could be kept for several weeks in the cold room at 0–2° C., or for several months in the frozen state at − 20° C. without deteriorating.

Incubations with fluorescent gamma globulin were 30 minutes in duration and were performed in test tubes agitated occasionally at room temperature. Preliminary studies showed nuclear uptake of gamma globulin to be essentially complete under these conditions. It was also found in preliminary studies that nuclear uptake of fluorescent gamma globulin [lupus serum L.E. (BO)] was proportional to nuclei over a wide range of nuclear additions (additions of nuclei equivalent to 90 to 900 mg. of liver), and was proportional to gamma globulin over a wide range of gamma globulin additions (0.1 to 4.0 mg.). For convenience the standard reaction mixture contained 0.1 ml. of rat liver (nuclei equivalent to 300 mg. of wet rat liver in buffered $CaCl_2$ sucrose), 0.1 ml. of buffered saline, and 0.05 ml. of conjugate. The protein content of the aliquot varied between 0.7 and 2.4 mg. from experiment to experiment, but in any particular experiment the protein of the control and the L.E. gamma globulin were quite similar (Table III). Following the incubation the excess conjugate was removed by washing the nuclei five times with 3.0 ml. of buffered $CaCl_2$ sucrose at room temperature. Prior to each centrifugation the suspended nuclei were permitted to stand five minutes at room temperature.

The uptake of fluorescent protein by the nuclei was determined by measuring the fluorescence of the washed nuclei in the Aminco-Bowman spectrophotofluorometer, using an exciting wave length of 365 mμ and measuring the fluorescence at 520 mμ. Because of the fluorescence of the turbid nuclear suspension itself, it was found desirable to scan each sample's emitted fluorescence spectrum from a wave length of 450 to 550 mμ, and to measure the height of the 520 mμ peak over the baseline fluorescence

TABLE II

L. E. cell formation after incubation of isolated nuclei with normal and L. E. gamma globulin, and after reincubation of the treated and washed nuclei with normal white blood cells

Expt.		Incubation		Reincubation	
		Control	L. E.	Control	L. E.
1	Nuclei from fresh buffy coat	0	0	0	470
2	Lyophilized leukemic nuclei			0	280
	Lyophilized leukemic mitochondria				0
3	Nuclei from fresh buffy coat	0	21	0	378

TABLE III

Uptake by nuclei of fluorescent gamma globulin from patients with disseminated lupus erythematosus and from normal controls

1 Expt.	2 Conjugate		3 Protein in aliquot	4 Fluoresc. in aliquot	5 Degree of fluoresc.	6 Corrected exptl. reading	7 Uptake exptl.	8 Gamma globulin of serum	9 Uptake calc.
no.	*no.*	*name*	*mg.*	*units*	*units/gamma protein*	*units*	*gamma/ mg. protein*	*mg. protein/ ml.*	*gamma/ ml. serum*
1	1.	Control (AA)	0.77	1,630	2.1	9	5.6	5.2	29
	2.	L. E. (BO)	0.67	2,020	3.0	119	59.2	26.3	1,560
2	3.	Control (JD)	2.2	2,870	1.3	17	5.9	4.7	28
	4.	L. E. (SU)	2.2	2,530	1.1	22	9 1	24.0	218
	5.	L. E. (VI)	2.1	2,980	1.4	54	18.4	16.7	307
	6.	L. E. (KR)	1.7	1,960	1.1	23	12.3	9.8	121
	7.	L. E. (MA)	2.4	3,260	1.4	145	43.2	16.3	705
3	8.	Control (WB)	2.0	3,880	1.9	23	6.1	4.9	30
	9.	L. E. (LE)	2.0	4,560	2.3	92	20.0	12.4	248
4	10.	Control (AA)	1.1	3,210	2.9	10	3.1	5.1	16
	11.	L. E. (TU)	1.9	7,410	3.9	221	29.8	14.5	432

(Figure 1). All measurements were performed in a 1.2 ml. volume using slit set No. 3, photomultiplier tube IP-28 at maximal or next to maximal sensitivity, and were recorded on a Bristol wide-strip recorder. On the Aminco-Bowman spectrophotofluorometer the potentiometer reading is a linear function of the fluorescence. An arbitrary fluorescence unit was defined as the amount of fluorescence in 1.2 ml. of a turbidity-free medium which would cause a 1 per cent potentiometer fluctuation at 520 mμ when excited at 365 mμ with the photomultiplier functioning at maximal sensitivity. The turbidity of the nuclear suspensions absorbed about two-thirds of the fluorescence, but to determine this absorbtion accurately, a correction factor was obtained by comparing the observed fluorescence of a standard aliquot of conjugate in 1.2 ml. of wash solution and in 0.1 ml. of nuclei diluted to 1.2 ml. with wash solution. An individual correction factor was obtained for each preparation of nuclei and for preparations of nuclei altered in various ways; this factor was used to correct the observed fluorescence. All the values recorded in the tables have been corrected in this manner. Since the fluorescence remains stable after dissolving the nu-

TABLE IV

Specific inhibition of nuclear uptake of fluorescent-labeled lupus gamma globulin

Expt.	Pretreatment source of unlabeled globulin	Source of labeled globulin	Experimental uptake	
			units	*gamma*
1	NaCl	Control (AA)	15	7.1
	NaCl	L. E. (BO)	122	40.7
	Control (AA)	L. E. (BO)	125	41.7
	L. E. (BO)	L. E. (BO)	28	9.3
	L. E. (MA)	L. E. (BO)	90	30.0
	L. E. (KR)	L. E. (BO)	120	40.0
2	NaCl	L. E. (BO)	76	25.3
	L. E. (BO)	L. E. (BO)	20	6.7
	L. E. (VI)	L. E. (BO)	105	35.0
	NaCl	L. E. (LE)	92	38.6
	L. E. (LE)	L. E. (LE)	33	13.8
	L. E. (BO)	L. E. (LE)	73	30.6
	NaCl	L. E. (VI)	72	51.1
	L. E. (VI)	L. E. (VI)	30	21.3
	L. E. (BO)	L. E. (VI)	66	46.8
	NaCl	Control (AA)	5	2.4

TABLE V

Uptake of fluorescent-labeled lupus gamma globulin by nuclei after pretreatment with desoxyribonuclease and trypsin

Expt.	Pre-treatment	Uptake (gamma) Control (AA)	Uptake (gamma) L. E. (BO)	DNA Klett units (E_{400}–E_{460})
1	Control	3.2	38.0	73
	DNAse	9.4	47.0	5
2	Control	6.7	57.9	
	DNAse		47.0	
	Trypsin		7.0	

clei in 1 N NaOH, the absorbtion measurements could be checked by adding NaOH. (The fluorescence is lost in acid solution, but recovered on neutralization.)

Tabulation of the uptake of fluorescent normal and L.E. gamma globulin was done in the following manner (Table III): The protein content (mg.) and the total fluorescence (units) of the 0.05 ml. aliquots of the various conjugates are reported in Columns 3 and 4 of this table, and the degree of fluorescence of the conjugates (units per gamma protein) are reported in Column 5. Column 6 presents the experimentally observed corrected fluorescence of nuclei (units) incubated with conjugate in the standard manner. In Column 7 this uptake of gamma globulin has been converted to gamma protein taken up per mg. protein in the aliquot by dividing the value in Column 6 by the degree of fluorescence (Column 5) and by the protein content of the aliquot (Column 3). The uptake per ml. serum (Column 9) is obtained by multiplication of the per mg. uptake (Column 7) by the concentration of gamma globulin of the original serum (Column 8).

Quantitative studies on the specificity of the nuclear staining reaction (Table IV) were performed by first incubating nuclei with 0.2 ml. of various unlabeled gamma globulin preparations, washing them twice at 0° C. and then reincubating them with labeled conjugate in the standard manner. The uptake in gamma is obtained by dividing the uptake in units by the degree of fluorescence of the appropriate conjugate.

Two types of quantitative studies on the nature of the nuclear component reacting with the lupus gamma globulin were done. In the first type (Table V), nuclei equivalent to 300 mg. of tissue were suspended in 0.3 ml. of buffered saline and pretreated for one hour at 30° C. with 0.5 mg. of desoxyribonuclease (DNAase) or trypsin in the presence of 0.005 M $MgCl_2$. The nuclei were then washed twice to free them of enzyme and incubated under standard conditions with fluorescent gamma globulin. Controls were pretreated with saline in the absence of enzyme.

The second type of experiment on the nature of the reacting component of the nucleus was done in a somewhat different manner (Table VI). Multiple duplicate aliquots of nuclei were incubated under standard conditions with the same fluorescent lupus gamma globulin and the initial uptake measured in the usual way. The labeled nuclei were then treated in one of the following ways: treated for 2 hours at 30° C. after suspending in 0.3 ml. of buffered saline with 0.5 mg. of DNAase or 0.2 mg. of ribonuclease (RNAase) in the presence of 0.005 M $MgCl_2$; extracted twice at 0° C. for 30 minutes with 1.2 ml. of 0.5 per cent citric acid in 1 M NaCl; or extracted three times at 0° C. over a 24 hour period with 1.2 ml. of 1 M NaCl. In each case the extracted fluorescence and the fluorescence of the residue was measured, and a control tube was run in which the nuclei were treated under identical conditions with buffered saline.

Nuclear fluorescence was frequently checked with a fluorescence microscope employing a Reichert 200 watt mercury vapor lamp and housing, a dark field condensor, and a filter system which passed light of 365 mμ wave length. Transient drying of nuclei was found to result in a marked loss of fluorescence.

Miscellaneous. The desoxyribonucleic acid (DNA) content of nuclei was determined with diphenylamine in the Klett colorimeter, using dichromatic readings (E_{400} to E_{650}) to reduce nonspecific color (16). A micromodification of the Kjeldahl procedure was used for the determination of protein concentration (17). DNAase, RNAase and trypsin were recrystallized materials obtained from the Worthington Corporation. Fluorescein amine was obtained from the Sylvania Chemical Company, Orange, N. J., and was converted to the isocyanate by the method of Coons and Kaplan (9). Other chemicals were commercial products of reagent grade. All experiments were performed with gamma globulin preparations from at least two different lupus patients.

RESULTS

Accessory serum factor

Table I presents data indicating that in addition to the L.E. factor, a second serum factor present in normal serum is necessary for the L.E. phenomenon. It can be seen in Experiments 1 and 2,

TABLE VI

Release of fluorescent lupus gamma globulin bound to nuclei by various treatments of the nuclei

Expt.	Treatment	Initial	Extract	Residue
		gamma	*gamma*	*gamma*
1	Control	30.0	2.3	20.3
	DNAse	30.0	1.0	21.0
2	Control	35.0	1.0	31.7
	0.5% citric acid in 1 M NaCl	35.0	0.7	27.7
3	Control	41.3	5.7	42.3
	1 M NaCl	41.3	29.0	12.7
4	Control	38.7		27.0
	RNAse	38.7		23.3

(in the absence of added $MgCl_2$) that washed white cells incubated with heated gamma globulin give a negative or weakly positive L.E. test. In contrast to this result, strongly positive L.E. tests are obtained when 0.1 ml. of normal serum is added. It is necessary to heat the gamma globulin to 56° C. to clearly demonstrate the requirement for normal serum factor. An accessory serum factor was previously described by Kurnick (18) who ascribed to it a role in the DNAase, DNAase-inhibitor system.

Under the conditions of the present experiments, heating the normal serum factor to 56° C. for 30 minutes reduced the activity to 29 per cent of that of the unheated control, and exposure to 0.03 N NH_4OH for 90 minutes at 37° C. reduced the activity to 27 per cent. However, activity of the combined heat-inactivated and NH_3-inactivated sera (19) was only 30 per cent. The failure to obtain complete activity from the combined inactivated sera suggests that the accessory serum factor is not identical with serum complement, but comprehensive studies in this regard have not been done. However, it is felt that this accessory serum factor acts as a phagocytosis-promoting factor similar to that described by Tullis (14). The evidence for this is given in Experiment 3 of Table I where studies on the phagocytosis of neutral starch granules in the presence and absence of normal serum factor are presented. It can be seen that washed leukocytes do not phagocytose starch granules in the presence of heated L.E. gamma globulin, while phagocytosis is marked when normal serum is added.

Table I also contains data on the effect of the addition of $MgCl_2$ and of DNAase on L.E. cell formation. $MgCl_2$ addition causes an increase in the number of L.E. cells in both the system with and the system without added normal serum factor, while the addition of DNAase results in a complete inhibition of L.E. cell formation.

Evidence of an initial reaction of nuclei with the L.E. gamma globulin prior to the phagocytosis of the nuclear material is presented in Table II. In these experiments, nuclei, after treatment with normal or L.E. gamma globulin, were washed to remove excess globulin, and then reincubated with normal serum and leukocytes. Nuclei treated with L.E. gamma globulin and then exposed to white cells and normal serum in a medium free of

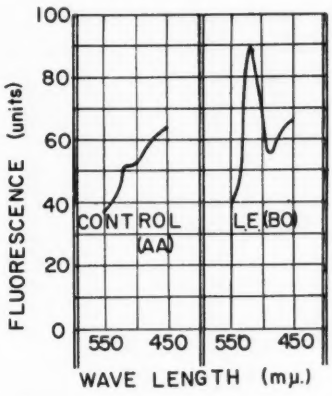

FIG. 1. REPRODUCTION OF AMINCO-BOWMAN SPECTROPHOTOFLUOROMETER TRACING OF EMISSION SPECTRUM FROM 450 TO 550 Mμ OF LIVER NUCLEI AFTER TREATMENT WITH FLUORESCENT GAMMA GLOBULIN FROM NORMAL CONTROL (AA) AND LUPUS PATIENT L. E. (BO)

L.E. factor went on to L.E. cell formation, while numerous controls, including untreated nuclei, nuclei treated with normal gamma globulin, and mitochondria treated with L.E. globulin, did not show L.E. cell formation. The small number of L.E. cells found after the initial incubation in Experiment 3 is caused by the few undisrupted cells (5 to 10 per cent) which are present in the preparations of fresh nuclei from normal buffy coat.

This then suggests that the accessory serum factor is necessary for the phagocytosis of the altered nuclear material (nuclear material which has reacted with L.E. gamma globulin) by normal leukocytes. That this extracellular nuclear material is actually altered by its reaction with L.E. factor is suggested by its morphologic appearance. Wright's stain of the control incubation without L.E. gamma globulin demonstrates a considerable amount of extracellular nuclear material (Feulgen positive) which is fine, lacelike and irregular in appearance (Figure 2). In the presence of heated L.E. gamma globulin, but without added normal serum, the nuclear material is dense, globular, purplish and homogeneous (Figure 3). This latter appearance is characteristic of the extracellular nuclear material and intracellular inclusions produced by L.E. sera as described by others (12, 20), but in the present situation the evolution of the L.E. cell is prevented by the absence of the phagocytosis-promoting factor.

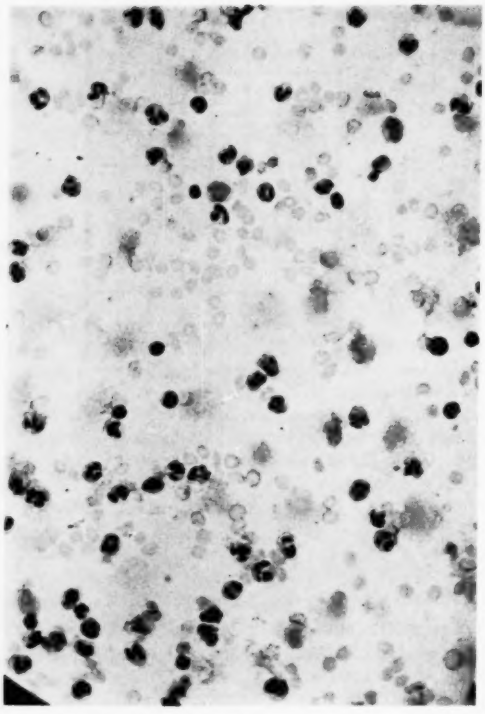

FIG. 2. EXTRACELLULAR NUCLEAR MATERIAL OBTAINED BY SHAKING WASHED WHITE BLOOD CELLS WITH GLASS BEADS IN THE PRESENCE OF ISOTONIC SALINE

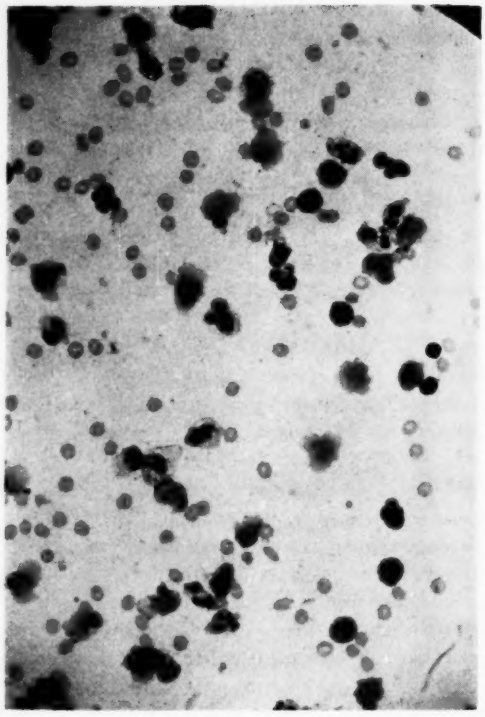

FIG. 3. EXTRACELLULAR NUCLEAR MATERIAL OBTAINED BY SHAKING WASHED LEUKOCYTES WITH GLASS BEADS IN THE PRESENCE OF HEATED LUPUS GAMMA GLOBULIN

Quantitative studies with fluorescent lupus globulin

Quantitative studies on the uptake by rat liver nuclei of fluorescent gamma globulin from four normal controls and seven patients with L.E.D. are presented in Table III. The maximum uptake of the controls is 6.1 gamma per mg. protein, while the lupus conjugates vary from 8.9 to 59 gamma per mg. The highest uptake of the lupus sera represents an uptake of 5.9 per cent of the protein of the aliquot. The gamma globulin uptake by nuclei when expressed per ml. of serum (Column 9) shows a more striking difference between L.E. patients and controls. This uptake does not exceed 30 gamma per ml. for the controls, while L.E. patients vary from 0.12 to 1.5 mg. per ml. serum.

Table IV presents quantitative studies on the specificity of the nuclear staining reaction performed by preincubating the nuclei with unlabeled gamma globulin. In Experiment 1 of Table IV it can be seen that specific inhibition of the uptake of labeled L.E. (BO) globulin is obtained by pretreatment with labeled globulin from this patient but not by pretreatment of the nuclei with normal gamma globulin or gamma globulin from two other lupus patients. To be certain that the difference in the inhibition of labeled L.E. (BO) uptake by the unlabeled gamma globulin preparations of the various L.E. patients used in Experiment 1 is not merely a reflection of a greater quantity of L.E. factor in the blood of L.E. (BO), Experiment 2 was done in which labeled and unlabeled gamma globulin from several patients with L.E. were reciprocally tested. It can be seen that the uptake of each patient's labeled gamma globulin is inhibited by prior treatment with unlabeled material from that patient, but not by unlabeled gamma globulin from other lupus patients.

Tables V and VI are concerned with studies on the nature of the nuclear component reacting with the lupus gamma globulin. In both Experiments

1 and 2 of Table V it can be seen that pretreatment of nuclei with DNAase, sufficient to remove more than 93 per cent of the DNA of the nuclei, does not reduce the subsequent uptake of fluorescent lupus gamma globulin by nuclei. Trypsin treatment does reduce the uptake of gamma globulin, but since trypsin completely destroys nuclear morphology this observation must be interpreted with caution (DNAase does not alter nuclear morphology).

In the experiments presented in Table VI, the nature of the reacting nuclear component was approached by studying the release of nuclear-bound lupus gamma globulin by various treatments of the nuclei. Experiment 1 confirms the above observation with DNAase, since there is no release of fluorescent material by the action of this enzyme on fluorescent gamma globulin-labeled nuclei. (The removal of DNA was checked as in Table V by direct determination after DNAase treatment.) In Experiment 2 of Table VI treatment with 0.5 per cent citric acid in 1 M NaCl fails to remove appreciable fluorescence, while Experiment 3 shows that repeated extractions with 1 M NaCl in the cold over a 24 hour period removes about two-thirds of the fluorescence bound to nuclei. The extracted fluorescent gamma globulin is still bound to the nuclear component in the extract, since dialyzing the 1 M NaCl extract against isotonic NaCl results in the formation of a fluorescent precipitate and a supernatant solution which is free of fluorescence. (In Experiment 3 of Table VI 40 per cent of the extracted fluorescence, all contained in the precipitate, could be recovered after dialysis.)

DISCUSSION

The present work supports the idea that a two-stage process is involved in L.E. cell formation. The first stage, specific for serum of patients with L.E., involves the reaction of a component of the cell nucleus with the L.E. factor and the morphologic alternation of the nucleus associated with this reaction. This first step is facilitated by rotation with glass beads, or in the present experiments, by presenting to the system isolated nuclei obtained by cell fractionation. The second stage in the L.E. phenomenon is the phagocytic step in which the altered nuclear material is phagocytosed by normal white cells to give L.E. cells. This step involves a heat-labile factor present in normal serum, believed to be a phagocytosis-promoting cofactor, but does not require L.E. gamma globulin. This two-stage formulation is consistent with the work of others (*20–22*), and certain of the present studies on this aspect of the L.E. phenomenon are similar to the work of Holman and Kunkel (8).

The present studies with fluorescent-labeled L.E. gamma globulin differ in two significant respects from the studies of other investigators (3–6). First, while in the past a fluorescent anti-gamma globulin rabbit serum was used, in the present work lupus gamma globulin was labeled directly. Secondly, because of the nature of the studies that were contemplated, a quantitative technique was developed. Aside from the disadvantage of requiring separate conjugation of each serum studied, the technique employed in the present work has one other important limitation.

Because absorption of the conjugate with liver powder removes some specific nuclear fluorescence, this step had to be omitted in the present studies. This has resulted in nuclei showing some uptake of control gamma globulin which repeated efforts have failed to eliminate. Furthermore, alterations in nuclei tend, in general, to enhance this non-specific uptake (Table V). The result of this limitation is that for the more refined measurements, such as the nature of the reacting nuclear component, only the most strongly reacting lupus conjugates can be studied with conclusive results.

Nonetheless interesting information on the L.E. phenomenon has been obtained by the present quantitative technique. First, it has been possible to demonstrate that nuclei show a greater uptake of L.E. than of normal gamma globulin, and to obtain a quantitative estimate of the amount of L.E. globulin reacting (Table III). Secondly, the specific inhibition studies bring out the interesting conclusion that the L.E. factor in various patients is not identical, since inhibition of labeled gamma globulin uptake by nuclei is only obtained by prior treatment of the nuclei with unlabeled gamma globulin from the same patient. Presumably, this difference of the L.E. factor in various patients is minor, since the experience of other investigators indicates that the L.E. factor reacts with anti-serum to normal gamma globulin (3–6) and has physical properties similar to normal

gamma globulin (2). Löw and Zetterström, employing an agar diffusion technique, also found minor differences in the L.E. factor in various patients (23). It is not unlikely that the major part of the L.E. factor molecule is identical with normal gamma globulin, while a minor part differs from normal gamma globulin and varies from patient to patient. Further, it is possible that the varied clinical picture of L.E.D. is a reflection of the difference in the L.E. factor in various patients.

The present studies suggest that the L.E. factor reacts with the nonhistone protein rather than the DNA of the nucleus. DNAase treatment fails to alter the reactability of nuclei with L.E. gamma globulin, and L.E. gamma globulin is not removed when fluorescent globulin-labeled nuclei are treated with DNAase. Extraction of histone with 0.5 per cent citric acid in 1 M NaCl (8) from the fluorescent globulin-labeled nuclei fails to extract fluorescence, while 1 M NaCl alone does extract the lupus globulin-nuclear component complex. This 1 M NaCl extract is composed largely of DNA, histone and nonhistone protein (24). It is of interest that the lupus globulin is evidently tightly bound to the nuclear component, since the complex survives 1 M salt extraction and subsequent dialysis.

The above evidence that the L.E. factor reacts with the nonhistone protein rather than with the DNA is in disagreement with the work of a number of other investigators (3, 4, 8, 25, 26). With regard to this point, the possibility exists in the present studies that artifacts may be caused by the development of nonspecific absorption by nuclei following enzyme treatment (Table V). Furthermore, because of considerations presented earlier, only the few most strongly reacting lupus sera could be studied with regard to the reacting nuclear component, and the results obtained may therefore not be generally applicable to lupus sera. It is of interest in this regard that Robbins, Holman, Deicher and Kunkel (25) have reported that certain lupus sera fix complement in the presence of whole nuclei, but not in the presence of DNA.

SUMMARY AND CONCLUSIONS

1. Studies on the lupus erythematosus (L.E.) phenomenon have shown that in addition to L.E. factor, nuclei, and normal leukocytes, an accessory serum factor present in normal serum is necessary for the L.E. phenomenon. This accessory factor is believed to be necessary for the phagocytosis of nuclear material which has been specifically altered by reaction with L.E. factor.

2. The reaction of fluorescent labeled lupus gamma globulin with nuclei has been quantitatively studied. Evidence for the heterogeneity of the L.E. factor in various patients has been obtained.

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EARLY EFFECTS OF THYROTROPIN ON PROTEIN-BOUND IODINE^{131} AND PLASMA IODIDE^{131} *

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The mechanism by which the pituitary thyroid-stimulating hormone (TSH) causes the thyroid gland to concentrate iodide and to synthesize and release hormone has not been clearly defined. Whether it acts by selectively stimulating one of the steps in thyroidal iodine metabolism, following which there is a secondary increase in the other reactions, or acts by affecting all stages of iodine metabolism simultaneously through some basic general stimulus to the entire cell is still a matter of speculation. Some of the evidence favoring the latter thesis rests on the demonstration that TSH affects such fundamental biochemical processes of thyroid tissue as oxygen consumption (2) and phospholipid synthesis (3, 4) and on the well known fact that TSH is required for normal growth and maintenance of the thyroid gland. One of the major obstacles to the universal acceptance of this thesis has been the apparent temporal dissociation of the effects of TSH on thyroid hormone release and iodide uptake by the gland. Although some workers have noted that stimulus to uptake occurred (after an eight hour delay) without any significant hormone release (5), perhaps the majority of data favor the view that in the normal gland hormone release occurs before iodide uptake is stimulated (6, 7).

The present study was designed in an attempt to re-evaluate these time relationships of TSH effects simultaneously in man by following protein-bound iodine^{131} (PBI^{131}) and plasma iodide^{131} disappearance time as measures of hormone release and thyroidal uptake of iodide, respectively. In the course of these studies observations were made that may explain previous discrepancies in the time relationships of these TSH effects.

MATERIAL AND METHODS

Oral tracer doses (200 to 600 μc.) of carrier-free sodium iodide^{131} were given to the euthyroid patients. These subjects were free of endocrine disease but in the case of the patients receiving the larger doses, did have some longevity-threatening illness. The previously untreated hyperthyroid subjects were given similar doses of I^{131} prior to treatment with radioiodine. Samples of heparinized venous blood were drawn at varying intervals throughout the remainder of the study. A single intramuscular injection of a thyrotropic hormone preparation (Armour Laboratory, Thytropar®) was given to each subject either at 12 or 24 hours after the original dose of I^{131}. To some subjects a second oral dose of I^{131} was given to maintain measurable levels of iodide^{131} in the blood for a total of 72 hours.

The blood samples were centrifuged and a 3 ml. aliquot of plasma from each sample was placed in a vial and

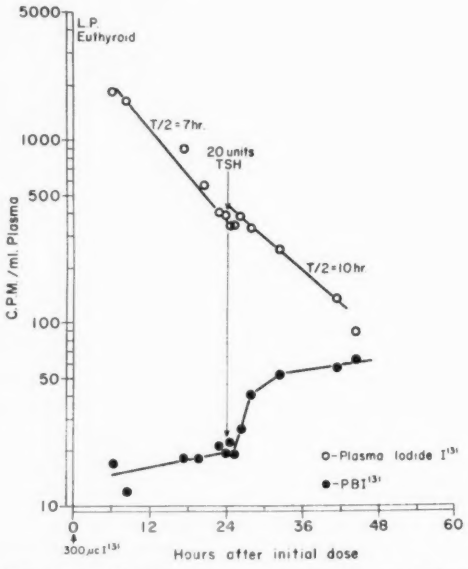

FIG. 1. PBI^{131} RELEASE AND SLOWING OF PLASMA IODIDE^{131} DISAPPEARANCE AFTER TSH ADMINISTRATION IN A NORMAL SUBJECT

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counted in a well-type scintillation counter equipped with a scaler and a pulse height analyzer. Background counts averaged 24 to 28 counts per minute (cpm). PBI^{131} was prepared by the ion exchange resin method of Fields and co-workers (8) and counted in the same scintillation well system. We have confirmed the validity of this resin method by recovering from the column 98 to 103 per cent of radiothyroxine (rechromatographed Abbott Laboratory preparation) added to plasma. Essentially all of the radioiodide added to serum is extracted by this resin. Values for PBI^{131} by this method were also in good agreement with those determined by trichloroacetic acid precipitation and by dialysis of plasma against physiological saline in all of the subjects' plasma so tested. All radioactivity measurements were corrected for physical decay to the time of the original dose of radioiodine. PBI^{131} was expressed as cpm per ml. The total plasma radioactivity minus the PBI^{131} was considered $iodide^{131}$.

RESULTS

Effects of TSH on plasma $iodide^{131}$ and PBI^{131} in euthyroid subjects

In Figures 1, 2 and 3 typical examples of simultaneous effects of TSH upon hormone release and plasma iodide are shown. The increase of PBI^{131} following TSH was prompt in each of these euthyroid subjects, occurring within 2 to 4 hours. In Figure 1 after a 6 hour equilibration period the control plasma $iodide^{131}$ had a disappearance halftime (T/2) of 7 hours. During the first 18 hours after 20 units of TSH intramuscularly the plasma iodide disappearance "slowed" to a T/2 of 10 hours. The lower plasma $iodide^{131}$ at 44 hours may be the first evidence of the speed-up in the thyroidal uptake seen in the subsequent figures. Figure 2 again shows the slowing of plasma $iodide^{131}$ disappearance from the control T/2 of 8.5 hours to 10.5 hours following TSH. In this study a second 50 μc. dose of NaI^{131} was given at 49 hours to increase the statistical significance of the radioactivity counts. Following 6 hours of equilibration the subsequent disappearance of the $iodide^{131}$ had increased to a T/2 of 7.0 hours which represents a slightly more rapid rate of disappearance than during the control period. A similar

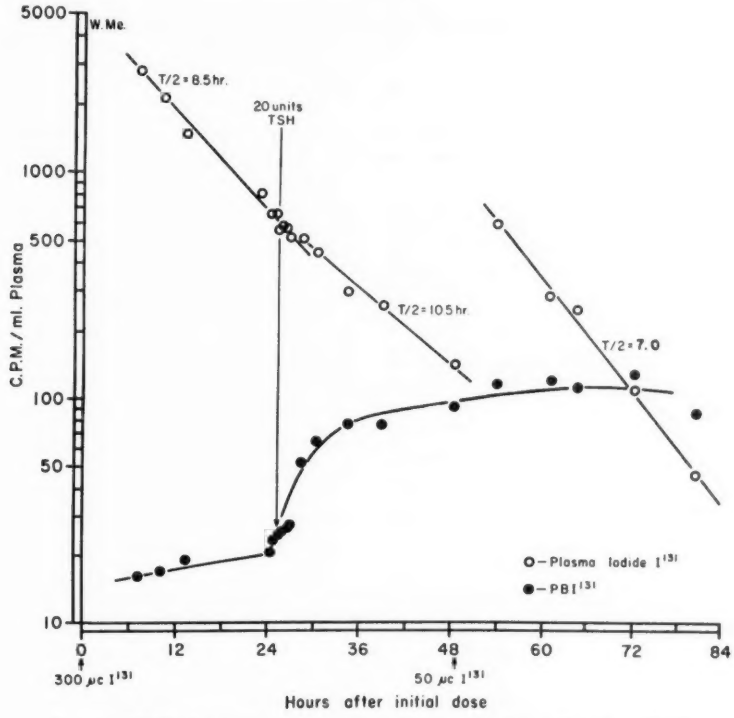

FIG. 2. PBI^{131} RELEASE AND CHANGES IN PLASMA $IODIDE^{131}$ AFTER TSH ADMINISTRATION IN A NORMAL SUBJECT

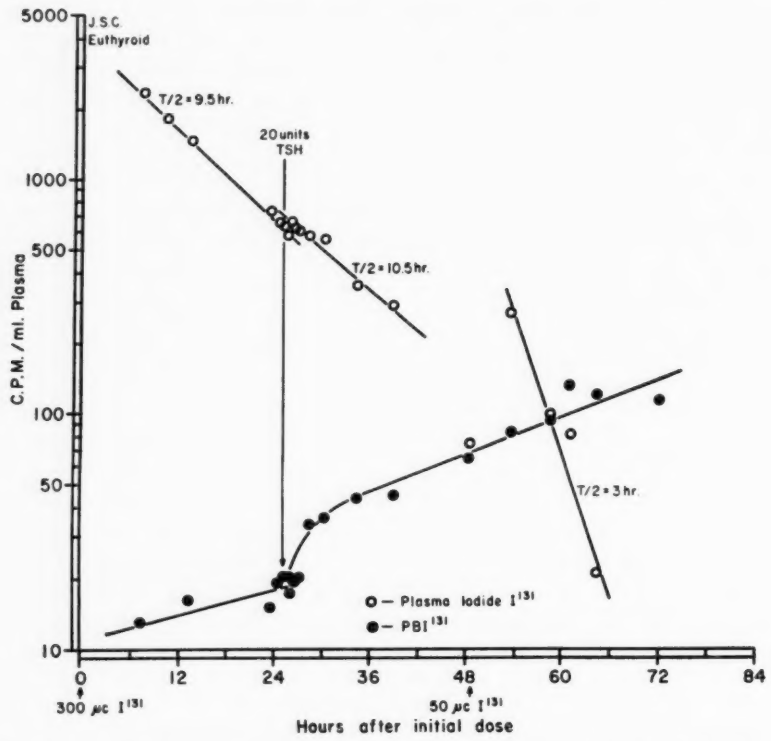

FIG. 3. INITIAL DECREASE AND SUBSEQUENT INCREASE IN PLASMA IODIDE^{131} DISAPPEARANCE AFTER TSH ADMINISTRATION IN A NORMAL SUBJECT

study is illustrated in Figure 3 where the plasma iodide^{131} disappearance slowed after TSH. By 48 hours (24 hours after the TSH) the plasma iodide^{131} content had begun to fall (note the single point at 48 hours just before the second dose of I^{131} was given). The very rapid disappearance of iodide thereafter is striking.

Table I is a summary of the results of comparable studies in the group of 17 euthyroid subjects. In all of these the PBI^{131} release by TSH was evident within 6 hours. During the first 12 to 24 hours after the TSH the plasma iodide disappearance was slowed from the mean control T/2 of 7.9 to 11.2 hours. It is quite significant that in four subjects there was either no further decrease or an actual increase in plasma iodide^{131}. During the period beginning 24 hours after the TSH administration the mean T/2 of the plasma iodide^{131} disappearance of 5.4 hours indicates that during this period the thyroidal uptake of plasma iodide had again accelerated.

When four additional subjects were given single 5 unit injections of TSH this slowing effect was not observed. Three of these patients showed a rise in PBI^{131} but no real change in plasma iodide^{131} disappearance was noted in 18 hours. This would lead to the conclusion that to observe this latter effect one must use a relatively large dose of TSH.

Effects of TSH on plasma iodide^{131} and PBI^{131} in hyperthyroid patients

Five patients with hyperthyroidism were studied in a similar fashion. Two of these studies are shown in Figures 4 and 5. In Figure 4 the control plasma iodide^{131} disappearance was rapid (T/2, 4 hours) as compared with the normal subjects, in keeping with the more rapid thyroidal clearance in such patients. Following 10 units

TABLE I

Effect of thyroid-stimulating hormone (TSH) on plasma iodide^{131} disappearance and protein-bound iodine (PBI)^{131} release in euthyroid subjects

No.	Patient	Dose 1	Control T/2 plasma iodide^{131} before TSH	TSH	T/2 plasma iodide^{131} during first 12–24 hours after TSH	Dose 2	T/2 plasma iodide^{131} from 24–48 hours after TSH	Time of definite stimulus after TSH on: PBI^{131} release	Time of definite stimulus after TSH on: Plasma iodide^{131} disappearance
		μc. I^{131}	*hours*	*units*	*hours*	*μc. I^{131}*	*hours*	*hours*	*hours*
1	J. W.	300	9.5	20	9.5			5	>18
2	E. A.	500	10.5	20	*		9.5	3	> 6
3	E. K.	500	8.0	20	8.0			3	>24
4	J. K.	600	9.0	20	9.0			1	>24
5	L. P.	300	7.0	20	10.0			4	>18
6	R. W.	300	6.5	20	11.0			2	>18
7	B. B.	300	8.0	20	21.0			4	10
8	R. V.	300	6.0	20	*			6	>18
9	L. W.	300	6.0	20	10.0	50	4.5	2	24
10	A. W.	300	10.5	20	10.5	50	4.0	2	33
11	H. C.	300	12.0	20	9.5	50	8.0	3	12
12	J. C.	200	7.0	20	14.0	50	4.0	6	31
13	J. P.	300	5.5	15	11.5	50	4.5	2	34
14	J. S. C.	300	9.5	20	10.5	50	3.0	3	23
15	W. Mc.	300	6.5	20	*	50	5.5	4	29
16	W. Me.	300	8.5	20	10.5	50	7.0	4	29
17	C. W.	300	5.0	20	*	50	4.0	2	10
Mean values			7.9		11.2		5.4		
p values					<0.01†		<0.01†		

* In these subjects there was either no further decrease or there was an increase in plasma iodide^{131} concentration.
† Values represent the difference between the means of paired data. Each of the periods was compared with the control period.

of TSH there was a distinct increase in plasma iodide^{131} lasting about 7 hours. On the day following the TSH plasma iodide was disappearing at a faster rate (T/2, 2.5 hours) than during the control period. It is difficult to say whether there was any increase in PBI^{131}. A very remarkable increase in plasma iodide^{131} followed 20 units of TSH given to the patient in Figure 5. In this subject as in several of the euthyroid group the plasma samples containing the increased plasma iodide^{131} after TSH were subjected to further tests to affirm the identity of this radioactivity as iodide. The same amounts of radioactivity (within experimental error) that remained on the resin column also were not precipitated by trichloroacetic acid and were dialyzable when the plasma samples were dialyzed against physiological saline. Butanol extracts of the plasma were chromatographed in butanol-ammonia and in collidine-water (9) and only thyroxine, triiodothyronine and iodide were found; *i.e.,* no iodotyrosines. Of the other three patients with hyperthyroidism one showed a response to the 20 unit dose very similar to that in Figure 5. One other subject showed some increase in iodide^{131} after 10 units of TSH followed by a marked slowing of iodide^{131} disappearance from a control T/2 value of 4 hours to a T/2 of 12 hours which persisted for 24 hours. These four patients all received their injections of TSH at 12 hours after the original dose of radioiodine for two reasons. The first of these was one of expedience since the plasma iodide^{131} falls so fast as a function of rapid thyroidal clearance that statistically significant counts would be difficult to obtain after 24 hours following a radioiodine dose in the tracer range. The second reason was that, since the intrathyroidal turnover of the isotope is greatly accelerated, the labeling of the thyroglobulin of the hyperthyroid patients at 12 hours would be more closely comparable to that in the euthyroid subjects at 12 hours. The remaining hyperthyroid patient was, however, given an injection of 20 units of TSH at 24 hours. While an increase of PBI^{131} was noted promptly, a very rapid increase in plasma iodide^{131} disappearance was noted.

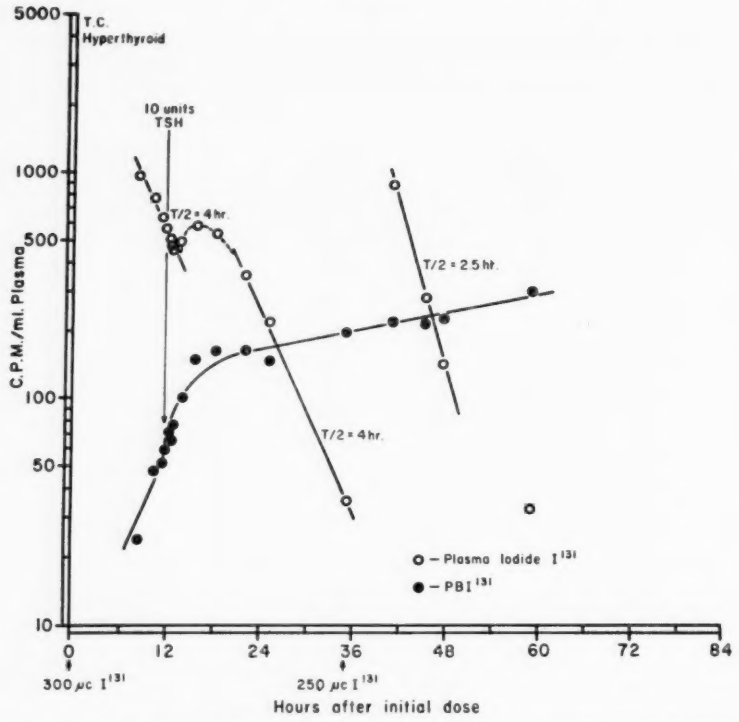

FIG. 4. TSH EFFECTS ON PBI^{131} AND PLASMA IODIDE^{131} IN A HYPERTHYROID SUBJECT

To test the possibility that the more marked effect of plasma iodide in the hyperthyroid patients was due to giving the TSH at 12 rather than at 24 hours after the dose of radioiodine two more euthyroid subjects were given 20 units of TSH at 12 hours after the I^{131}. In one there was no response of either the PBI^{131} or the plasma iodide^{131}. In the other, however, a modest increase of PBI^{131} and a slight slowing of plasma iodide^{131} disappearance were noted. This would indicate that the timing of the TSH administration alone is not sufficient explanation for the more marked response in the hyperthyroid patients.

DISCUSSION

The rapid increase in PBI^{131} following these doses of TSH was expected but prolongation of plasma iodide^{131} disappearance time and, in some experiments, actual increase in plasma iodide^{131} is, of course, not what one would anticipate if TSH were causing early stimulation to the trapping mechanism with an increase in thyroidal clearance rate of plasma iodide. Before attempting an explanation of these changes in plasma iodide produced by TSH, it is appropriate to inspect the validity of the plasma iodide^{131} disappearance time as a measure of thyroidal uptake and utilization under the conditions of these studies.

After equilibration the disappearance of plasma iodide^{131} is a function of both renal and thyroidal clearances. Therefore to use the plasma iodide^{131} disappearance time as a measure of thyroidal uptake following TSH we must assume that any early effect on the plasma iodide^{131} is a result of thyrotropin activity on its primary target, the thyroid, rather than upon renal clearance, extrarenal extrathyroidal iodide space, or peripheral degradation of endogenously labeled hormonal iodine. That these are reasonable assumptions can be supported in part. The early effect of TSH on renal clearance of iodide^{131} (using endogenous creatinine for glomerular filtration rate) was negligible when

calculated in two normal subjects given 20 units of TSH 24 hours after the dose of radioiodine. That the early effects of TSH on plasma iodide^{131} cannot be attributed to alterations in renal clearance was further shown when these same effects, *i.e.*, actual increase in plasma iodide^{131}, were produced in a patient with acute renal failure whose urine output was only 100 ml. per day. The only experimental evidence we have that the effects are not due to some alteration of extrarenal, extrathyroidal iodide space was the failure to detect any change in plasma iodide^{131} with TSH in a patient given sufficient inorganic iodide to block thyroidal uptake of the tracer dose of radioiodide. That increase in peripheral degradation of endogenously labeled thyroxine by TSH cannot account for the early changes in plasma iodide^{131} is evident from the fact that repeated doses of TSH (5 units every 24 hours for a three day period) given to five patients whose thyroids were blocked by potassium iodide did not alter the biologic decay of radiothyroxine administered before the TSH. Even though the fractional rate of peripheral degradation of hormone remains constant in spite of wide variations in circulating hormone (10) it is conceivable that the increase in specific activity of the endogenously labeled hormone released by TSH would lead to an increase in plasma iodide^{131} arising from peripheral deiodination of this hormone. This iodide^{131} would then invalidate the use of the plasma iodide^{131} disappearance time as a measure of thyroidal uptake. It is not likely that this is a problem during these studies since in many patients the plasma iodide^{131} increased as much as did the PBI^{131} in the first three to four hours after TSH. This would be far in excess of the usually quoted figure of 10 per cent degradation per 24 hours. To check this possibility further, however, two normal subjects were given high specific activity radiothyroxine (that had been added to their plasma *in vitro* and dialyzed against physiological saline to remove any iodide^{131}) intravenously in amounts sufficient to simulate the PBI^{131} curves after TSH. Plasma samples were drawn thereafter and analyzed as in the TSH studies. There was essentially no evidence of "deiodination" iodide^{131} in any of the samples during the three hours of these studies. Comparable studies with radiotriiodothyronine showed less than 10 per cent of the total plasma

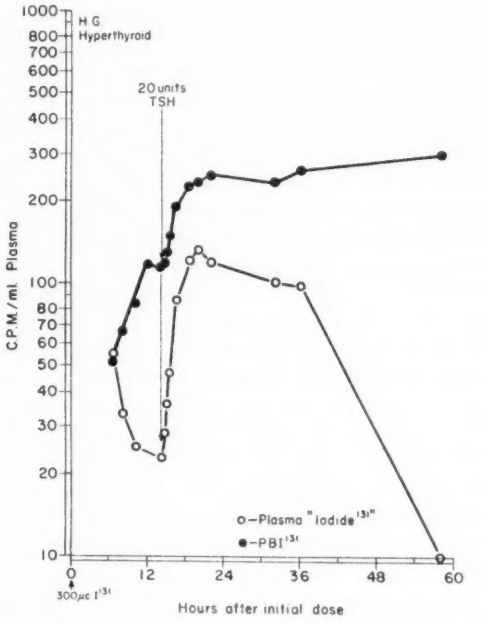

FIG. 5. MARKED INCREASE IN PLASMA IODIDE^{131} AFTER TSH ADMINISTRATION IN A HYPERTHYROID SUBJECT

counts at any time as iodide^{131}. The PBI^{131} in the labeled triiodothyronine studies was determined by dialysis of plasma samples since this hormone is less firmly bound to protein and adheres to the anion exchange column usually used. One further possibility to account for the increase in plasma iodide^{131} is that iodotyrosines were released by TSH and deiodinated sufficiently rapidly (11) to contribute to plasma iodide. Though this cannot be absolutely ruled out we were never able to detect increase in iodotyrosine in plasma chromatographs.

It is a reasonable assumption then that the early slowing of the plasma iodide^{131} disappearance time and in some instances the increase in plasma iodide^{131} results from the effect of thyrotropin on the thyroid and only after this phase is it possible to detect the increased trapping of plasma iodide by these methods. These observations suggest that some product of the TSH-increased proteolysis of thyroglobulin temporarily acts as an inhibitor of further utilization of iodide, *i.e.*, an intrathyroidal feedback. This inhibition may affect the iodide being recycyled from the deiodinase reaction within

the gland as well, producing in some patients the actual increase in plasma iodide^{131} from leakage of this iodide to the plasma. This loss of intrathyroidal "deiodinase" iodide is compatible with the data of Nadler and Leblond (12) who find that not all the trapped iodide is oxidized and that some of the recycled iodide from the deiodinase reaction also is exposed to re-entry to the plasma.

This interpretation of our data in the human is also consistent with experiments of Halmi (13) who found in rats an initial depression of the thyroid to serum (T/S) ratio of iodide^{131} following TSH before the increase in T/S was noted. This same interpretation fits equally well with data of others (14, 15) showing an actual depression of *in vitro* uptake of iodide^{131} by isolated thyroids of normal rats treated with TSH whereas hypophysectomized rats (presumably thyroglobulin-depleted) similarly treated showed increased uptake. These observations may also explain the differences in rate of iodide uptake as compared with hormone release noted in the literature (5, 16) for if the thyroglobulin deficient gland is stimulated by TSH one would anticipate less early inhibition and consequently the uptake of plasma iodide would be greater. Conversely, the more adequately thyroglobulin-stocked the gland, the more delayed the trapping of the administered iodide. Halmi has described in animals an intrathyroidal "depressor" mechanism operating to control uptake of iodide (13). Our data can be interpreted indirectly to support the existence of such a mechanism in man.

Unfortunately, it is not possible to say whether or not the biochemical mechanism responsible for trapping is stimulated as promptly as is the hormone secretion, for it is still possible that it is simultaneously stimulated but temporarily blocked by the postulated product of thyroglobulin proteolysis, or flooded with deiodinase iodide. The latter then must either be reutilized or released before the effect of the stimulation can become manifest as increased trapping of plasma iodide. It does seem, however, that we should beware of emphasizing, as a strong point in favor of the view that TSH primarily affects secretion, the observation that increased hormone release precedes stimulated uptake of the administered dose of iodide, since study of the trapping mechanism soon after TSH will be confused by the release of intrathyroidal iodide.

The striking increase in plasma iodide^{131} following TSH in three of the five hyperthyroid patients would seem to indicate that in these patients the loss of intrathyroidal iodide is greater after TSH than in normal subjects. Regarding the possible positive (17) or negative (18) role of TSH in the etiology of Grave's disease, all that can be said from our data is that it can be shown that TSH can produce in some euthyroid subjects the excessive loss of intrathyroidal iodide to the plasma that is reported (19, 20) to occur in untreated patients with Grave's disease.

SUMMARY AND CONCLUSIONS

Following the administration of radioiodine to 17 euthyroid subjects plasma iodide^{131} disappearance and protein-bound iodine^{131} were determined as measures of thyroidal uptake of iodine and thyroid hormone release, respectively. Following a single injection of thyrotropin prompt release of protein-bound iodine^{131} was noted before six hours in all subjects. During the first 12 to 24 hours after thyrotropin there was a significant increase in plasma iodide^{131} disappearance time in 12 subjects. In 4 of these there was a period during which plasma iodide^{131} either did not decrease further or actually increased. It was only after this period of slowed iodide disappearance that the more rapid iodide^{131} disappearance was observed.

These changes in plasma iodide^{131} are believed to reflect an intrathyroidal feedback mechanism activated by some product of the thyrotropin-induced increase in thyroglobulin proteolysis or by increased deiodinase activity leading to a period of decreased plasma iodide disappearance and/or to leakage of intrathyroidal iodide^{131} to the plasma. These indirect effects of thyrotropin upon the thyroid trap make interpretation of a possible direct effect of this tropic hormone on iodide trapping difficult. These observations should lead, however, to a cautious approach in assigning the primary locus of thyrotropin activity to thyroglobulin proteolysis simply because hormone release can usually be detected before increased uptake of iodide in normal subjects.

Similar studies were made on five hyperthyroid subjects. In one of these a prompt increase in

iodide^{131} disappearance was noted. In one patient a marked decrease was seen and in the other three there was a pronounced loss in intrathyroidal iodide^{131} into the plasma. The possible implication of these observations is briefly discussed.

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EFFECTS OF ISOPROTERENOL ON PERIPHERAL VENOUS TONE AND TRANSMURAL RIGHT ATRIAL PRESSURE IN MAN *

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The increase in cardiac output during isoproterenol administration has been attributed to a direct action on the heart causing it to extract blood more effectively from the central venous reservoir (1). Another suggestion is that isoproterenol constricts peripheral veins and causes blood to be pushed into the heart (2). The observation that right atrial pressure falls during infusion of the drug (3) would seem to dispose of the latter explanation. Right atrial pressure, measured with respect to atmospheric pressure, however, is not the force which distends the atrium. The pressure which actually distends the atrium, the *transmural* pressure, is the difference between the pressure within the chamber and the intrapleural pressure which surrounds it. Transmural atrial pressure, then, may be influenced by changes in intrapleural pressure.

In previous studies (4) we found that hyperventilation causes transmural atrial pressure to rise even though atrial pressure falls. This occurs because overbreathing is associated with a greater reduction in intrapleural pressure than in right atrial pressure. Isoproterenol, like epinephrine, appears to cause central excitation (5). It is known that epinephrine causes hyperventilation (6), probably by a central excitatory action. If isoproterenol has a similar effect on breathing, the fall it produces in right atrial pressure could be a ventilatory effect. If this were true the fall in atrial pressure might not indicate a coincident fall in transmural pressure.

If isoproterenol causes blood to be pushed centrally, peripheral venous constriction would have to provide the "pushing" force. To our knowledge no observations on the peripheral venous responses to isoproterenol have been reported. Since epinephrine causes venous constriction in the forearm (7) and since some actions of isoproterenol on the vascular system are different from those of epinephrine (5), the matter of isoproterenol causing venous constriction and a shift of blood from the periphery of the body needs investigation.

This report concerns experiments which were done to see what happens to peripheral venomotor tone and transmural right atrial pressure during isoproterenol administration.

METHODS AND PROCEDURES

The experiments were performed on 10 healthy male medical students. In the first group of five experiments subjects were studied in the right lateral decubitus position with the right arm extended downward through a hole in the table. Right atrial pressure was measured with a Statham 0 to 5 cm. Hg strain gage connected to a needle in the antecubital vein of the dependent arm according to the method of Gauer and Sieker (8). Intrapleural pressure was measured as esophageal pressure with a Statham 0 to 5 cm. Hg strain gage connected to a small, open-ended, water-filled polyethylene tube. The tip was placed in the lower third of the esophagus. Esophageal pressure is similar to but not identical with intrapleural pressure. Changes in the two tend to be parallel (9), however, so that esophageal pressure changes reflect changes in intrapleural pressure. The difference between right atrial and esophageal pressure, the *transmural* right atrial pressure, was measured with a Sanborn differential pressure transducer. End-expiratory CO_2 concentration was monitored with a Liston-Becker CO_2 analyzer and ventilation was measured with a gas meter (10). All three pressures and CO_2 concentration were recorded simultaneously with a Sanborn direct-writing oscillograph.

After control values were established isoproterenol chloride [1] was infused into a foot vein at a constant rate of 5.8 μg. per minute. Experimental observations were made after the pressures had been stable for at least 45

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[1] Isuprel®, Winthrop Laboratories, New York, N. Y.

seconds. In most cases the measurements were made 90 to 120 seconds after the infusion was started. The infusion usually was discontinued as soon as the experimental measurements were obtained. Observations were continued until control values returned. Reported mean pressures were obtained by counting squares under the curves registered by electrical integration of the output of the manometers. The sensitivity of the amplifying system was adjusted so that respiratory pressure changes when integrated electrically occupied at least 2 to 3 cm. on the recording paper. In most experiments 1 cm. vertically on the recording paper was equal to 2.0 or 5.0 mm. Hg. Pressures were read to the nearest 0.5 mm. Hg.

In the second group of five experiments the subjects were studied in the supine position. Forearm venous pressure-volume curves were obtained by a modification (11) of the plethysmographic method of Litter, Wood and Wilkins (12, 13). Forearm volume changes were measured by means of two partially immersed electrodes (14) which sensed changes in the height of the water in a vertical cylinder attached to the top of the plethysmograph. Except for this change in the measurement of volume the pressure-volume curves were obtained as in previous experiments with epinephrine (7). Forearm venous pressure was measured in the antecubital vein of the arm unencumbered by the plethysmograph. In all experiments the arm in which venous pressure was measured was below atrial level. Curves and venous pressure measurements were obtained before, during and after isoproterenol infusion into a foot vein as in the first group of experiments.

Following the experiments, venous pressure-volume curves were constructed. These curves (Figure 1) express in ml. per 100 ml. of forearm tissue the volume to which the veins are distended by any level of transmural venous pressure between 0 and 30 mm. Hg. The final point on the curve, the venous volume at 30 mm. Hg, is called arbitrarily the *venous distensibility*. A high value represents venous dilatation and a low value venous constriction. Assuming that the venous pressure-volume characteristics are the same in both arms, the naturally occurring venous volume of the forearm is the volume coordinate of that point on the curve which corresponds to the natural venous pressure (equals transmural venous pressure) measured in the arm opposite the one in the plethysmograph. The venous volume existing during each measurement of distensibility was determined by drawing a line perpendicular to the pressure axis from the natural venous pressure value (Figure 1). If a stimulus such as isoproterenol produces venous constriction and also a fall in venous pressure, the decrease in volume caused by each of these factors alone may be determined. The volume coordinate of the point on the isoproterenol curve which corresponds to the control venous pressure is the venous volume which would have existed had the total volume reduction been caused by venous constriction alone. The difference between this value and the venous volume which existed during the stimulus represents the reduction in venous volume caused by the fall in venous pressure alone.

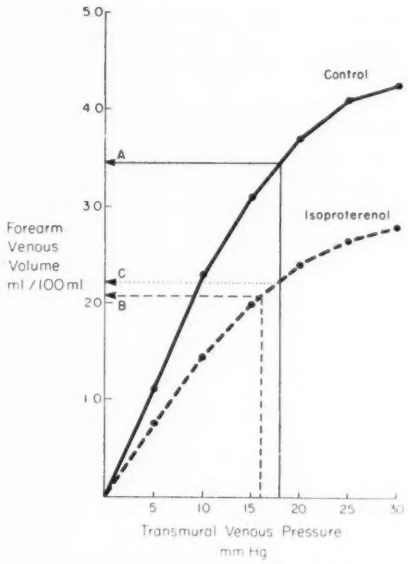

FIG. 1. VENOUS PRESSURE-VOLUME CURVES (EXPERIMENT 3)

A refers to the forearm venous volume which existed during the control period. B refers to the volume during isoproterenol infusion. C refers to the volume which would have existed during isoproterenol infusion if there had been only venous constriction and no change in venous pressure.

Statistical analysis of the data was carried out according to methods described by Fisher (15).

RESULTS

Effect of isoproterenol on end-expiratory CO_2 tension and ventilation

Ventilation increased in each of five experiments during isoproterenol infusion (Table I). The average increase was 8.0 L. per min. ($p < 0.01$). End-expiratory CO_2 tension decreased in each experiment. The average decrease was 5.8 mm. Hg ($p < 0.01$).

Effect of isoproterenol on transmural right atrial pressure

Despite the hyperventilation no consistent changes were observed in mean esophageal pressure. It increased in two, decreased in one and remained unchanged in two of the five experiments

TABLE I

Transmural right atrial pressure responses to isoproterenol infusion

Experiment	Esophageal pressure		Atrial pressure		Transmural atrial pressure		End-expiratory CO_2 tension		Ventilation	
	Control	Isoproterenol	Control	Isoproterenol	Control	Isoproterenol	Control	Isoproterenol	Control	Isoproterenol
no.	*mm. Hg*		*mm. Hg*		*mm. Hg*		*mm. Hg*		*L./min.*	
1	−7.0	−6.0	4.0	3.0	11.5	9.5	42.0	38.1	6.6	11.1
2	−7.0	−7.0	3.0	2.0	9.0	8.5	36.2	33.2	4.1	13.6
3	−2.5	−2.5	−0.5	−3.0	2.5	−0.5	43.0	34.0	7.5	14.7
4	−7.5	−9.5	4.5	1.0	12.5	11.5	45.8	39.5	6.8	18.8
5	−8.0	−7.0	3.0	1.5	11.0	8.0	42.3	35.6	4.1	10.8
Mean difference		0.0		1.9		1.9		5.8		8.0
Standard error		0.55		0.48		0.51		1.07		1.30
Probability		>0.9		<0.02		<0.02		<0.01		<0.01

(Table I). The average difference between esophageal pressures measured during control periods and during drug infusion was zero. Right atrial pressure, however, fell during isoproterenol administration in each experiment. The average decrease was 1.9 mm. Hg ($p < 0.02$). The transmural right atrial pressure also fell in each experiment. The average decrease was 1.9 mm. Hg ($p < 0.02$). The fall in transmural pressure resulted from the fall in right atrial pressure since esophageal pressure was essentially unchanged.

Forearm venous responses to isoproterenol infusion

Forearm venous pressure fell in four and remained unchanged in one of five experiments during isoproterenol infusion (Table II). The average change was 1.8 mm. Hg ($p < 0.05$). Venous distensibility (the venous volume at a transmural venous pressure of 30 mm. Hg) decreased in each of the five tests. The average decrease was 1.5 ml. per 100 ml. of forearm tissue ($p < 0.001$). The naturally occurring forearm venous volume averaged 3.8 ml. per 100 ml. during control periods and fell to an average of 2.2 ml. per 100 ml. during isoproterenol infusion. This shift of blood from the extremity averaged 1.5 ml. per 100 ml. or 39.5 per cent of the average control value ($p < 0.001$). The amount of blood pushed from the forearm by venous constriction alone averaged 1.3 ml. per 100 ml. or 34.2 per cent of the control value ($p < 0.001$). The amount which left the arm simply because of the fall in distending pressure averaged 0.2 ml. per 100 ml. or 5.3 per cent of the control volume.

The changes in transmural right atrial pressure, venous distensibility and venous pressure were maintained as long as the infusions were con-

TABLE II

Forearm venous responses to isoproterenol infusion

Experiment	Venous pressure		Venous distensibility		Venous volume		
	Control	Isoproterenol	Control	Isoproterenol	Control	Isoproterenol	Isoproterenol*
no.	*mm. Hg*		*ml./100 ml.*		*ml./100 ml.*		
1	17.5	16.0	5.3	3.6	4.0	2.3	2.5
2	22.5	20.0	5.1	3.4	4.5	2.8	3.1
3	18.0	16.0	4.2	2.8	3.4	2.2	2.4
4	20.0	17.0	3.5	2.2	3.1	1.5	1.7
5	20.0	20.0	4.3	3.1	3.8	2.4	2.4
Mean difference		1.8		1.5		1.5	1.3
Standard error		0.51		0.10		0.10	0.09
Probability		<0.05		<0.001		<0.001	<0.001

* Forearm venous volume which would have existed during isoproterenol infusion if there had been only venous constriction and no change in venous pressure.

tinued. The infusion periods ranged from four to nine minutes.

DISCUSSION

In previous experiments (16) venous constriction occurred during voluntary hyperventilation. This suggests that the constriction observed in the present investigation also might be attributed to overbreathing. The venous constriction associated with isoproterenol, however, greatly exceeded that observed with voluntary hyperventilation despite greater ventilatory rates and larger reductions in end-expiratory CO_2 tension in the earlier experiments. The venous constriction seen with isoproterenol administration probably cannot be explained on the basis of hyperventilation alone.

If the peripheral venous constriction observed in these experiments were generalized the net result would be a shift of blood centrally. There is some support for this suggestion in the work of Bruce, Cobb, Katsura and Morledge (17) who reported increases in central blood volume with isoproterenol infusion in certain patients. A central shift of blood might seem inconsistent with the fall in transmural atrial pressure which we observed. The pressure in the central venous system, which may be considered a direct extension of the atrium, however, depends not only upon the volume of blood within it but also on the tonic state of its walls. If isoproterenol causes increased distensibility or reduced tone of the central veins the pressure could be low even though the volume were large.

The presence of increased cardiac output during isoproterenol administration (1, 18) might appear inconsistent with the decreased mean transmural atrial pressure if one accepts this parameter as an index of ventricular filling pressure. Sarnoff's concept of "a family of ventricular function curves" (19) seems useful in explaining the situation. According to his view increased stroke work in the presence of reduced filling pressure is still compatible with the Starling relationship between atrial pressure and cardiac output. The relationship is simply shifted to a higher curve, *i.e.*, a level of function which yields more stroke work for a given pressure. This appears to occur with epinephrine (19) and it might occur with isoproterenol. A shift to a higher curve with these agents might result from increased contractility (leading to greater systolic emptying) or greater diastolic distensibility of the myocardium. In either case ventricular filling could increase even though filling pressure fell. The concept of increased myocardial distensibility has considerable support from intact dog (20) and isolated heart (21) experiments in the case of epinephrine whose other actions on the myocardium are similar to those of isoproterenol (5).

These experiments show that venous constriction shifts blood from the periphery and that transmural atrial pressure falls with isoproterenol. Despite this apparent paradox the fall in transmural pressure does not exclude a central shift of blood from consideration as an important determinant of the increased cardiac output. It tends to reinforce the thesis (1) that the action of isoproterenol on the myocardium does something to cause output to increase.

SUMMARY

Right atrial, intrapleural (esophageal) and transmural right atrial pressure were recorded simultaneously along with end-expiratory CO_2 concentration before and during intravenous infusion of isoproterenol. Ventilation was measured intermittently. Despite the mild hyperventilation which occurred in each experiment, intrapleural pressure changes were not significant. Atrial pressure fell regularly as did the transmural pressure. The fall in transmural pressure was caused by the fall in atrial pressure because intrapleural pressure was not changed appreciably. The fall in atrial pressure previously observed (3) probably is not a ventilatory effect.

In other experiments forearm venous distensibility and pressure were measured before and during isoproterenol infusion into a foot vein. Venous pressure fell in most tests but venous constriction occurred regularly. The large shift of blood from the forearm which occurred in these experiments was caused primarily by the venous constriction and not by the fall in intraluminal pressure.

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THE INFLUENCE OF URINARY IONIC STRENGTH ON PHOSPHATE pK_2' AND THE DETERMINATION OF TITRATABLE ACID *

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It is well recognized that the pK′ value for the salt of a weak acid or base is significantly influenced by the ionic strength of the solution. In the case of phosphate, the theoretical value for pK_2 in an infinitely dilute solution is 7.181 at 37° C. (1) and in a solution of ionic strength comparable to that of plasma is approximately 6.8. The small variations in ionic strength ordinarily encountered in plasma would not be expected to produce significant deviations from this latter value. However, in view of the wider range of ionic strengths which occurs in urine, values differing appreciably from 6.8 might be anticipated.

In the present study an attempt has been made to evaluate the relationship between pK_2' phosphate and ionic strength in urine, and to define the usual range of values which might be encountered in the course of acute phosphate loading experiments. A method has been devised for the determination of phosphate pK_2' in urine and the experimental values have been compared with those for phosphate solutions of similar ionic strength. The data suggest that under the conditions of the present experiments the behavior of phosphate in urine conforms closely to that predicted for simple phosphate solutions by the Debye-Hückel equation. It has further been demonstrated that the pK_2' of phosphate in urine may vary significantly and that under some circumstances failure to consider this variation may introduce appreciable errors into either the measurement or calculation of urinary "titratable acid."

THEORETICAL CONSIDERATIONS

The dissociation constant of a weak acid may be determined with reasonable accuracy for dilute solutions by employing concentrations in the mass law equation. Thus, the value of K in the equation

$$(1) \qquad K = \frac{[H^+][A^-]}{[HA]}$$

remains constant over a wide range of concentrations. In the case of strong electrolytes or the salts of weak acids, the dissociation constant is not accurately predicted by the mass law equation if concentrations are employed. According to current theory, this deviation from ideal behavior is not the result of incomplete dissociation but is accounted for by changes in activities induced chiefly by interionic electrostatic forces. The variation from ideal behavior is expressed by the activity coefficient, γ:

$$(2) \qquad \gamma = \frac{a}{c},$$

where a is the observed activity and c, the concentration. In general, γ decreases with increase in concentration and approaches unity at high dilution. If $\gamma \cdot c$ is substituted in the mass law equation, the change in K can then be predicted. Thus for the reaction $H_2PO_4^- \rightleftarrows H^+ + HPO_4^=$, the following equation may be written:

$$(3) \qquad K_2 = \frac{a_{H^+} \cdot \gamma_2 \cdot [HPO_4^=]}{\gamma_1 \cdot [H_2PO_4^-]}.$$

In the logarithmic form, this becomes:

$$(4) \quad pK_2 = pH + \log \frac{[H_2PO_4^-]}{[HPO_4^=]} + \log \frac{\gamma_1}{\gamma_2}$$

or:

$$(5) \quad pK_2 - \log \frac{\gamma_1}{\gamma_2} = pH + \log \frac{[H_2PO_4^-]}{[HPO_4^=]} = pK_2'.$$

pK_2 is the theoretical value for an infinitely dilute solution where γ_1 and $\gamma_2 = 1$. At any finite

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concentration, however, the observed pK (pK_2') will vary from pK_2 by the term $\log \frac{\gamma_1}{\gamma_2}$.

The relationship between the activity coefficient of an ion and ionic strength, μ, of the solution may be expressed by the Debye-Hückel equation:

$$(6) \qquad -\log \gamma_i = \frac{A \cdot Z_i^2 \sqrt{\mu}}{1 + B\,a\sqrt{\mu}}.$$

Z_i is the valence of the ion. A and B are constants, dependent upon temperature and the solvent. a is the "interionic diameter." The ionic strength of the solution, μ, is calculated by multiplying the concentration of each ion, C_i, by the square of its valence Z_i^2 adding all such products, and dividing by 2. $\mu = \frac{\Sigma\, C_i\, Z_i^2}{2}$.

Substitution of 4.5 Å for a and the appropriate values for A and B (1) in Equation (6) yields an equation for each form of phosphate. The combination of these equations gives:

$$(7) \qquad \log \frac{\gamma_1}{\gamma_2} = \frac{1.57\sqrt{\mu}}{1 + 1.49\sqrt{\mu}}.$$

Equation (5) may then be rewritten:

$$(8) \quad pK_2 - \frac{1.57\sqrt{\mu}}{1 + 1.49\sqrt{\mu}} = pH + \log \frac{[H_2PO_4^-]}{[HPO_4^=]} = pK_2'.$$

In this form, the equation is valid to an ionic strength of about 0.1. For ionic strengths above this, an additional term must be added which has been found to be linear with respect to ionic strength (2). The expanded equation may be written:

$$(9) \quad pK_2 - \frac{1.57\sqrt{\mu}}{1 + 1.49\sqrt{\mu}} + \beta\mu = pH + \log \frac{[H_2PO_4^-]}{[HPO_4^=]} = pK_2'.$$

The value of pK_2 for phosphate solutions at 37° C., obtained by extrapolation to zero ionic strength, is approximately 7.181 (1). Thus if the ionic strength and ratio of phosphate are known and pH determined experimentally, the equation may be solved for β. The value of the linear constant, β, in the case of phosphate solutions is a function of the ratio of the two forms of phosphate (3). Bates and Acree (1) have studied the second dissociation constant of sodium phosphate solutions in which the fractions of total phosphate as $HPO_4^=$ were approximately 0.4, 0.5 and 0.6. These workers employed molal concentrations and measured E.M.F., using hydrogen and silver chloride electrodes and a technique which eliminated errors due to liquid junction potential. Since the method commonly employed in biologic work is pH measurement with the glass electrode, these constants have been restudied for sodium phosphate solutions at 37° C. using the glass electrode. Furthermore, molar concentrations have been employed in the present study in order to obtain results which could be compared more closely with those of urine where concentrations are ordinarily measured and expressed on a molar basis. In addition, linear constants have been determined for a wider range of phosphate ratios than studied by Bates and Acree, in order to encompass the range found in urine. No attempt was made to correct for liquid junction potential error in the pH measurement of either phosphate solutions or urine.

Within certain limits, the Debye-Hückel equation also predicts changes in activity coefficients for solutions containing more than one electrolyte. In dilute solutions, the activity coefficient of a given strong electrolyte is the same in all solutions of the same ionic strength (4). Thus in an equimolar phosphate buffer solution whose ionic strength is increased from 0.1 to 0.2 by the addition of sodium chloride, the change in activity coefficient is simply that predicted by the change in ionic strength (2). The effects of higher concentrations of NaCl have not previously been studied, nor have data been available on the influence of NaCl or other salts when phosphate is present in other than equimolar concentrations.

METHODS

A. Experimental determination of pK_2' phosphate and linear constants in simple solutions

1. Sodium phosphate solutions. Standard solutions were made up so that the ratio of $H_2PO_4^-$ to $HPO_4^=$ was varied over a range from 19/1 to 0.43/1. For convenience, these ratios may be expressed as fractions of total phosphate present as $HPO_4^=$ and will be referred to as mole fractions in the remainder of this paper. The mole fractions, corre-

sponding to the above ratios, ranged from 0.05 to 0.70 and were achieved by individual weighings of Na_2HPO_4 and NaH_2PO_4. All solutions were made up in triplicate to a final molarity of 0.50.

The following procedure was carried out in duplicate with each standard solution: Successive 1 ml. aliquots were added from a buret to a beaker containing 10 ml. of distilled H_2O. By this means the molarity of the solution in the beaker was increased in a step-wise fashion from 0.0455 to 0.2500. The pH was determined after each increment. Substitution of pH and the appropriate values for μ and $\log \frac{[H_2PO_4^-]}{[HPO_4^=]}$ in Equation (*9*) was then made and the equation solved for β.

2. Sodium phosphate solutions with added salts. The effects of increasing quantities of NaCl and Na_2SO_4 on the pK_2' of phosphate was studied by utilizing 50 and 100 mM sodium phosphate solutions of mole fractions, again ranging between 0.05 and 0.70. NaCl or Na_2SO_4 was added to a portion of each phosphate solution in quantities sufficient to make the final concentrations of these two salts 0.3 and 0.2 M, respectively. Successive 1 ml. aliquots of these latter solutions were then added from a buret to a beaker containing the comparable phosphate solution unmodified by addition of chloride or sulfate. In this way, the concentration and ratio of the two forms of phosphate was held constant while the concentration of the salt being studied was progressively increased. pH was measured after each 1 ml. increment and, from appropriate substitutions in Equation (*9*), β was calculated.

B. Experimental determination of pK_2' phosphate in urine

Twelve experiments were performed on 11 female dogs anesthetized with 1 per cent chloralose. Urine composition was varied by infusion of sodium phosphate solutions (4 to 1 ratio of $HPO_4^=$ to $H_2PO_4^-$) at different rates or by addition of NaCl or Na_2SO_4 to the infusing solution. When phosphate alone was used, the infusate was brought to isotonicity by addition of glucose.

Since pH = pK_2' when $[H_2PO_4^-] = [HPO_4^=]$, pK_2' may be determined by measurement of pH when equimolar concentrations of phosphate have been established. This goal can be achieved experimentally if essentially all phosphate is first converted to $H_2PO_4^-$ by addition of acid and one-half of the phosphate then titrated to $HPO_4^=$ by addition of alkali. At a pH of 4.4 approximately 99 per cent of phosphate is present as $H_2PO_4^-$ over the widest range of pK_1' and pK_2' values which might be expected to occur in urine. If this pH lay exactly half-way between pK_1' and pK_2', the concentrations of H_3PO_4 and $HPO_4^=$ would be equal and therefore equivalent in buffer capacity to an equimolar quantity of $H_2PO_4^-$. However, the error introduced by ignoring the deviation from this ideal relationship in the subsequent determination of pK_2' is, by calculation, less than 0.01.

The following experimental procedure was employed: Aliquots of each urine specimen were acidified to a pH of 4.4 ± 0.1 with 1.0 N HCl. One-tenth N NaOH was then added in an amount equivalent to one-half of the total phosphate. An additional amount of alkali was added equivalent to organic buffer present between approximately pH 4.4 and 6.8. The pH after addition of alkali was taken as the value of pK_2'.[1]

The ionic strength of urine specimens after addition of alkali was calculated by correcting the original electrolyte concentrations for the change resulting from the addition of HCl and NaOH. $H_2PO_4^-$ and $HPO_4^=$ were assumed to be present in equimolar concentrations and organic anions present between pH 2.7 and 6.8 were included in the calculation and taken as univalent.

C. Chemical measurements

pH determinations were made with a Cambridge research model pH meter, using an external glass electrode and calomel reference electrode. All determinations were carried out in water bath maintained at 37 ± 1° C. The pH meter was standardized with National Bureau of Standards potassium acid phthalate (pH 4.025 at 37° C.), and the response of the entire assembly tested frequently by use of National Bureau of Standards sodium borate (pH 9.086 at 37° C.). Slight deviations from ideal response over this pH range were compensated for by adjustment of the temperature control mechanism (5). pH readings were made to the nearest 0.005 unit.

The following measurements were made on the urine by methods previously described (6): pH (anaerobic), sodium, potassium, ammonium, phosphate, chloride and CO_2 content. Inorganic sulfate was determined gravimetrically by the method of Folin (7). The sum of calcium and magnesium was measured by the method of Natelson and Penniall (8). Urines were prepared for organic acid titration by the method of Van Slyke and Palmer (9). In order to insure complete dephosphorylation, specimens were agitated for 30 minutes in an automatic wrist-action shaking device. The filtrates were acidified with 1 N HCl to pH 2.7 and titrated with 0.1 N NaOH. Titration readings were taken at pH 4.4, 6.8 and at the pH of the urine as excreted.

RESULTS

A. pK_2' and linear constants for sodium phosphate solutions

Tables I and II present the data for phosphate solutions in which the mole fractions were 0.5 and 0.1, respectively. As shown in Column 1 of Table I, the solutions ranged in molarity from 0.0455 to 0.2500. Columns 2 and 3 give the ionic strength and square root of ionic strength, respectively, and Column 4 presents theoretical values for pK_2' calculated from Equation (*8*). Each pH value appearing in Column 5 represents

[1] The same titration technique was applied to phosphate solutions of known ionic strength for the purpose of determining the accuracy of the method in simple solutions. Calculated and observed pK_2' values agreed within ±0.02 unit.

TABLE I

Experimentally determined pK_2' values and derived linear constants, β, for sodium phosphate solutions containing equimolar concentrations of $H_2PO_4^-$ and $HPO_4^=$

Molarity	μ	$\sqrt{\mu}$	$7.181 - \frac{1.57\sqrt{\mu}}{1+1.49\sqrt{\mu}}$	pH (pK_2')	$\beta\mu$	β
0.0455	0.0910	0.302	6.854	6.848	−0.006	−0.07
0.0833	0.1666	0.408	6.782	6.753	−0.029	−0.17
0.1154	0.2308	0.480	6.741	6.714	−0.027	−0.12
0.1429	0.2858	0.535	6.714	6.672	−0.042	−0.15
0.1667	0.3334	0.577	6.694	6.656	−0.038	−0.11
0.1875	0.3750	0.612	6.678	6.632	−0.046	−0.12
0.2059	0.4118	0.642	6.666	6.619	−0.047	−0.11
0.2222	0.4444	0.667	6.656	6.605	−0.051	−0.11
0.2368	0.4736	0.688	6.648	6.596	−0.052	−0.11
0.2500	0.500	0.707	6.640	6.575	−0.065	−0.13

the average of six determinations. In Table I these values are taken to be the experimentally determined pK_2' values, since $HPO_4^=$ and $H_2PO_4^-$ were present in equimolar concentrations. It can be seen that the experimentally determined pK_2' values differ from those calculated from Equation (*8*) by an amount which is a linear function of ionic strength [Equation (*9*)]. This difference, $\beta\mu$, is shown in Column 6 and the linear constant, β, in Column 7. The pK_2' values for this buffer ratio decreased from 6.848 to 6.575 as the ionic strength was increased from 0.091 to 0.500. β, however, remained relatively constant with an average value of −0.12.

Table II is similar to Table I in all respects except that pK_2' was determined by adding the logarithm of the phosphate ratio to the measured pH value. The difference between these pK_2' values and those calculated from Equation (*8*) are shown in Column 7 and the corresponding linear constant, β, in Column 8. pK_2' fell progressively from 6.877 to 6.570 over a range of ionic strength from 0.0546 to 0.300. β remained relatively constant and had an average value of −0.45.

In Figure 1 are shown the β values for all mole fractions studied. The best fitting curve has been drawn through the points by inspection. The values for β ranged from −0.51 for mole fraction 0.05 to −0.02 for mole fraction 0.7. It is apparent that the value rapidly becomes more negative as the ratio of acid to basic phosphate is increased.

By substitution of each value for β in Equation (*9*) a curve describing the relationship between pK_2' and ionic strength may be obtained for each mole fraction. Figure 2 shows the family of curves derived by this method. At low ionic strengths (less than 0.04) there is no appreciable difference in pK_2' for various mole fractions. However, as ionic strength is increased, the curves diverge. Thus at an ionic strength of 0.36, for example, pK_2' lies between 6.51 and 6.67.

TABLE II

Experimentally determined pK_2' values and derived linear constants, β, for sodium phosphate solutions containing a nine to one ratio of $H_2PO_4^-$ to $HPO_4^=$

Molarity	μ	$\sqrt{\mu}$	$7.181 - \frac{1.57\sqrt{\mu}}{1+1.49\sqrt{\mu}}$	pH	$\text{pH} + \log \text{R}$ (pK_2')	$\beta\mu$	β
0.0455	0.0546	0.234	6.909	5.923	6.877	−0.032	−0.59
0.0833	0.0999	0.316	6.844	5.852	6.806	−0.038	−0.38
0.1154	0.1385	0.372	6.805	5.799	6.753	−0.052	−0.38
0.1429	0.1715	0.414	6.779	5.755	6.709	−0.070	−0.41
0.1667	0.2001	0.447	6.760	5.713	6.667	−0.093	−0.46
0.1875	0.2251	0.474	6.745	5.682	6.636	−0.109	−0.48
0.2059	0.2471	0.497	6.733	5.667	6.621	−0.112	−0.45
0.2222	0.2666	0.516	6.723	5.645	6.599	−0.124	−0.47
0.2368	0.2842	0.533	6.714	5.627	6.581	−0.133	−0.47
0.2500	0.300	0.548	6.708	5.616	6.570	−0.138	−0.46

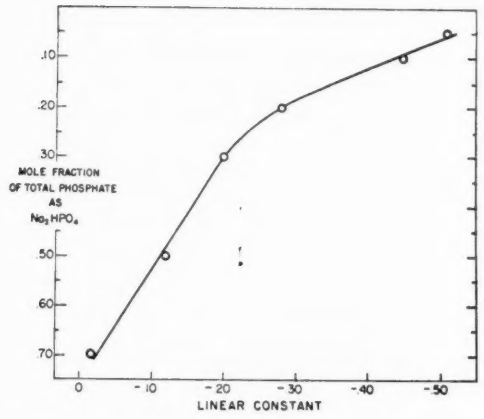

FIG. 1. RELATIONSHIP BETWEEN THE LINEAR CONSTANT, β, AND THE RATIO OF $HPO_4^=$ TO $H_2PO_4^-$

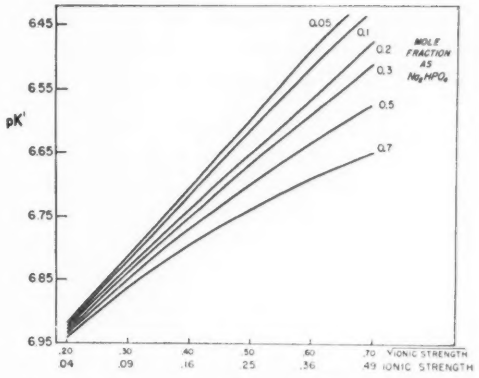

FIG. 2. RELATIONSHIP BETWEEN PHOSPHATE pK_2' AND IONIC STRENGTH

Each curve represents the pK_2' values for a given ratio of $HPO_4^=$ to $H_2PO_4^-$.

The addition of NaCl in quantities up to 100 mM per L. had no significant effect on the β values of 50 and 100 mM phosphate solutions over the entire range of mole fractions (0.05 to 0.70). At higher concentrations of NaCl, the value for β rose progressively, and thus β cannot be expressed as a single value at NaCl concentrations above 100 mM per L.

Na_2SO_4 in concentrations up to 100 mM per L. had no effect on the β value of equimolar phosphate solutions. In more acid phosphate solutions, however, Na_2SO_4 in concentrations from 20 to 100 mM per L. significantly affected the values for β, although the new values were essentially constant over this range of sulfate concentrations. For the mole fractions 0.05, 0.10, 0.20, 0.30 and 0.50 the values of $-\beta$ were 0.31, 0.25, 0.20, 0.17 and 0.12, respectively.

B. pK_2' of phosphate in urine

Table III shows the results of a typical experiment. The infusing solutions consisted of sodium phosphate plus glucose during Periods 1 through 4 and sodium phosphate plus saline during Periods 5 through 8. The electrolyte concentrations shown in the table have been corrected for the changes resulting from the titration procedure, and the ionic strength (μ) calculated from these concentrations.[2] The last two

[2] The sum of calcium and magnesium was measured in representative urines from each experiment and was found to be approximately 1 mM per L. in most specimens and in no instance greater than 2 mM per L. In view of these low concentrations, the effects of complex ion formation with divalent anions was ignored in both the experimental determination of pK_2' and the calculation of ionic strength. It is also recognized that the ionic strength calculated for

TABLE III

Effect of variation in urinary ionic strength on pK_2' phosphate during acute phosphate loading

Period	Na	K	NH_4	Cl	PO_4	Organic acid	μ	$\sqrt{\mu}$	pK_2' calculated	pK_2' observed
	mEq./L.	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mM/L.*	*mEq./L.*				
	Infusion of Na_2HPO_4, 40 mM/L.; NaH_2PO_4, 10 mM/L.; glucose, 30 Gm./L.									
1	55	27	18	7	44	27	0.1220	0.349	6.81	6.82
2	39	9	7	6	24	21	0.0710	0.266	6.88	6.91
3	39	5	5	10	19	19	0.0628	0.251	6.89	6.91
4	36	4	3	10	18	15	0.0565	0.238	6.90	6.91
	Infusion of Na_2HPO_4, 40 mM/L.; NaH_2PO_4, 10 mM/L.; NaCl, 500 mM/L.									
5	35	4	3	12	17	12	0.0543	0.233	6.90	6.91
6	61	6	4	25	26	7	0.0840	0.290	6.85	6.88
7	96	5	3	61	23	6	0.1143	0.338	6.81	6.79
8	138	5	2	100	23	6	0.1543	0.393	6.77	6.76

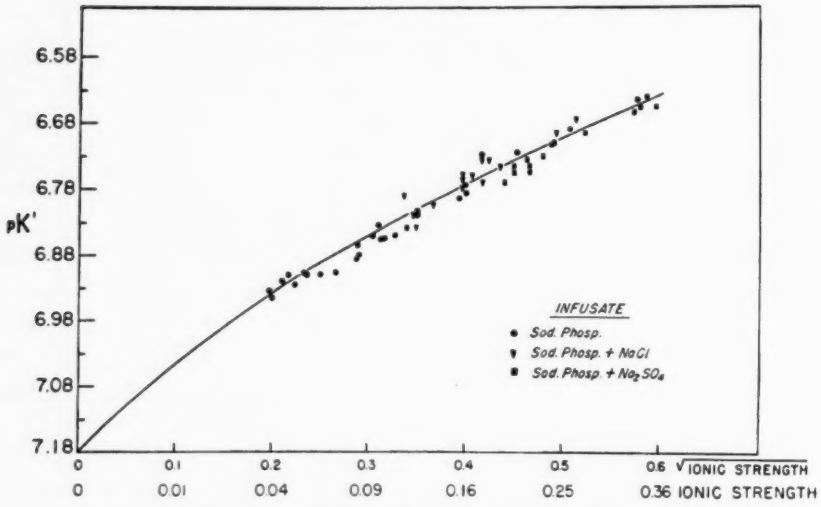

FIG. 3. EXPERIMENTALLY DETERMINED pK_2' VALUES FOR PHOSPHATE IN URINE

The experimental points have been plotted as a function of the calculated ionic strength of the urine. The solid line describes the relationship between pK_2' and ionic strength for equimolar sodium phosphate solutions and is described by Equation (9) with a β value of -0.12.

columns compare calculated and experimentally observed pK_2' values. These differed by ± 0.02 unit or less in six observations and by 0.03 in the remaining two. During the course of the experiment, there was a threefold variation in ionic strength and pK_2' varied between 6.91 and 6.76.

Figure 3 summarizes the results of all 12 experiments. The solid line describes the relationship between pK_2' and ionic strength for phosphate solutions of 0.5 M fraction. The observed pK_2' values have been plotted as a function of the estimated ionic strength of each specimen after titration. The range of observed values was from 6.95 to 6.64. The experimental points fell within ± 0.02 pK unit of the predicted values in 80 per cent of determinations and within ± 0.03 in the remainder.

The presence of chloride or sulfate up to a concentration of approximately 100 mM per L. had no apparent effect upon the agreement between observed and theoretical values.

urine is not exactly comparable to the ionic strength of a simple phosphate solution (when both calculations are based on molar concentrations) since substances other than phosphate present in urine alter the ratio of water to total solute. This slight discrepancy has also been ignored.

DISCUSSION

The data indicate that, under the present experimental conditions, the pK_2' of phosphate in urine changes as a function of ionic strength in a fashion similar to that observed in simple phosphate solutions and in accord with the predictions of Debye-Hückel theory [Equation (9)]. The experimentally determined pK_2' values ranged from 6.64 to 6.95. As in equimolar sodium phosphate solutions, the pK_2' was apparently uninfluenced by the presence of large amounts of chloride or sulfate, except to the extent that these ions contributed to the total urinary ionic strength.

The experimentally determined pK_2' values cannot be taken as those of the original urines, since the titration procedure employed in the measurement of pK_2' phosphate in itself changed the composition of the urine. The original values could be calculated if one knew both the initial urinary ionic strength and the phosphate ratio with its appropriate linear constant, β. However, β and the calculation of ionic strength depend on the ratio of the two forms of phosphate, a proportion which cannot be determined analytically. This problem can be circumvented,

and a close estimate of pK_2' obtained, by means of the following series of approximations:

1. The ratio of $HPO_4^=$ to $H_2PO_4^-$ is estimated from the Henderson-Hasselbalch equation, using the measured urine pH and an arbitrary pK_2' value of 6.8.

2. The ionic strength of the urine is calculated from the individual electrolyte concentrations, the contribution of phosphate being estimated from the ratio calculated in Step 1.

3. An appropriate β value is chosen from the phosphate ratio, and utilizing this and the ionic strength, a value for pK_2' is calculated from Equation (*9*). In the choice of an appropriate linear constant the possible influence of any other ion present in high concentration must be considered. In the case of chloride, it has been demonstrated that concentrations up to 100 mM per L. exert no significant effect on the β value of 50 and 100 mM sodium phosphate solutions over a wide range of buffer ratios. On the other hand, sulfate significantly modifies β except when the two forms of phosphate are present in equimolar concentrations (see Results).

4. The entire process of calculation is repeated, using the pK_2' value obtained in Step 3. The resulting pK_2' ordinarily is found to vary by less than 0.01 from that derived in the first series of calculations.

This method of approximation, when applied to phosphate solutions of known composition, yields results within ± 0.01 of the correct pK_2'. The pK_2' phosphate values for the urine as excreted, when estimated by this method, were found to lie between 6.56 and 6.98.

The pK_2' of phosphate in normal plasma, where ionic strength is estimated to be 0.167 (10), has generally been taken as approximately 6.8. Based on the behavior of phosphate in solutions where ionic strength, alkalinity and sodium chloride concentrations are comparable to plasma, this value appears to be reasonable. The variations in plasma ionic strength and pH ordinarily encountered *in vivo* would be expected to induce a shift in pK_2' of only several hundredths. Plasma thus stands in contrast to urine where pK_2' varies widely as a result of the much greater range in ionic strength and pH.

The variability of pK_2' in urine indicates that the ratio of $HPO_4^=$ to $H_2PO_4^-$ may differ appreciably in plasma (glomerular filtrate) and urine even when both solutions are at the same pH. Thus in the measurement of "titratable acid," it cannot be assumed that returning the urine pH to that of the plasma will necessarily restore the phosphate buffer ratio to that which was filtered at the glomerulus. To the extent that the ratio in the urine after titration to plasma pH differs from the ratio which existed in the filtrate, the estimate of renal acid excretion will be in error. Table IV illustrates the magnitude of errors which would result from assuming a pK_2' of 6.8 for urine if this value were actually 6.6. pK_2' of phosphate in plasma (and filtrate) has been taken as 6.8. The errors have been calculated by comparing the "titratable acid" obtained by returning the phosphate ratio to that in the glomerular filtrate (true acid excretion) with "titratable acid" obtained by titrating the urine to plasma pH (usual method). In Section A the plasma pH has been taken as 7.4 and in Section B, 7.1. It is apparent that regardless of the initial urine pH, the quantity of phosphate over-titrated is the same and the absolute error is therefore constant. However, the

TABLE IV

Errors in "Titratable Acid" (T.A.) resulting from use of 6.8 for pK_2' phosphate when true value is 6.6 *

Initial urine pH	Section A Blood pH, 7.4				Section B Blood pH, 7.1			
	Apparent T.A. at pH 7.4	True T.A. $\frac{HPO_4}{H_2PO_4} = \frac{4}{1}$	Absolute error	Per cent error	Apparent T.A. at pH 7.1	True T.A. $\frac{HPO_4}{H_2PO_4} = \frac{2}{1}$	Absolute error	Per cent error
	mEq./L.	*mEq./L.*	*mEq./L.*		*mEq./L.*	*mEq./L.*	*mEq./L.*	
5.0	83.9	77.6	+6.3	+ 8.1	73.6	64.2	+9.4	+ 14.6
6.0	66.1	59.8	+6.3	+10.5	55.8	46.4	+9.4	+ 20.3
6.4	47.7	41.4	+6.3	+15.2	37.4	28.0	+9.4	+ 33.6
6.8	24.9	18.6	+6.3	+33.9	14.6	5.2	+9.4	+180.8

* Calculations based on urine phosphate concentrations of 100 mM per L.

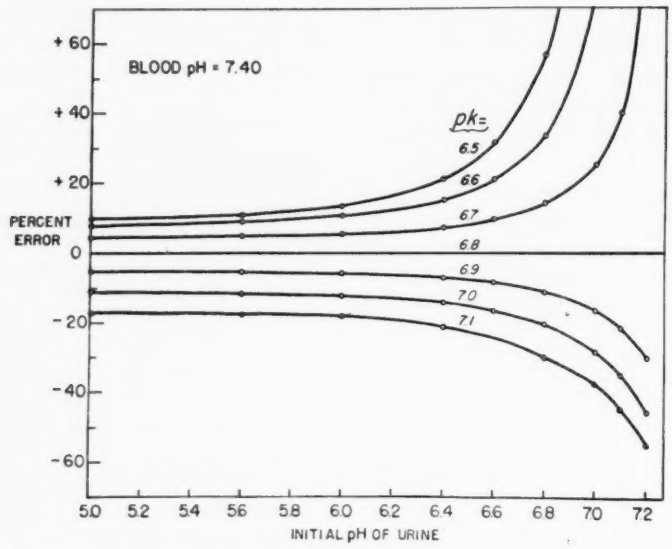

FIG. 4. PER CENT ERRORS IN "TITRATABLE ACID" RESULTING FROM USE OF AN ASSUMED pK_2' PHOSPHATE OF 6.8 IN URINE RATHER THAN THE TRUE VALUE

On the abscissa are shown the initial pH values of the urine. Blood pH has been taken as 7.4.

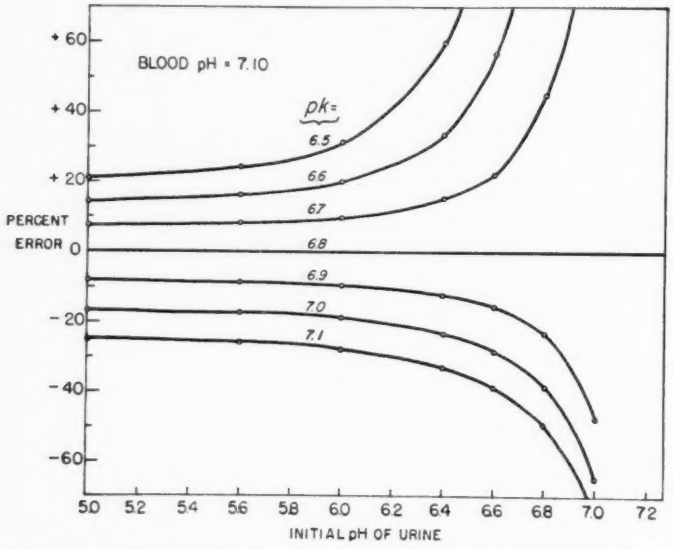

FIG. 5. PER CENT ERRORS IN "TITRATABLE ACID" RESULTING FROM USE OF AN ASSUMED pK_2' PHOSPHATE OF 6.8 IN URINE RATHER THAN THE TRUE VALUE

On the abscissa are shown the initial pH values of the urine. Blood pH has been taken as 7.1.

per cent error is a function of total "titratable acid" and thus is greater with higher initial urine pH values. When plasma pH is lower than normal, the errors in "titratable acid" are larger. Thus, when the same urines are returned to a plasma pH of 7.1, the absolute error is greater and the per cent error for each starting pH is also significantly increased.

Figures 4 and 5 show the per cent errors in "titratable acid" which are introduced by assuming a pK_2' of 6.8 when the true values range between 6.5 and 7.1. In Figure 4, the plasma pH has been taken as 7.4 and in Figure 5, as 7.1. On the abscissa is shown initial urine pH and on the ordinate the positive or negative error expressed as a per cent of the true "titratable acid." Each curve thus describes the errors for a given pK_2' phosphate over a wide range of initial urine pH values. Between pH 5 and 6, the errors are relatively small but increase in a hyperbolic fashion between pH 6 and 7. As illustrated in Table IV, the errors are significantly larger when acidosis is present.

Regardless of the original pK_2' of phosphate in the urine, dilution of the specimen prior to titration will elevate pK_2' above the initial value. For example, a 1 : 5 dilution causes an upward shift in pK_2' of 0.1 to 0.2 unit. In the case of urines of high ionic strength and low pK_2' phosphate, dilution might fortuitously shift pK_2' to a value near 6.8 and thus reduce the error in "titratable acid" which would have occurred if urine pH had arbitrarily been returned to that of plasma. However, a 1 : 5 dilution shifts any initial pK_2' phosphate of 6.7 or above to values between 6.90 and 7.10 and a 1 : 10 dilution shifts pK_2' phosphate of even the most concentrated specimens to this same range. The magnitude of the resulting errors in the measurement of "titratable acid" is shown in Figures 4 and 5.

Close agreement between measured "titratable acid" and a value calculated from the Henderson-Hasselbalch equation, utilizing a pK_2' of 6.8, has often been observed during phosphate loading (11, 12). This agreement would have resulted if the urine pK_2' phosphate values were, in fact, approximately 6.8. However, it can be readily shown that all phosphate buffer solutions with pK_2' values between 6.6 and 7.0 have approximately the same buffer capacity when they are titrated from an initial pH between 5.6 and 6.6 to a final pH between 7.1 and 7.4. Thus, agreement with a calculated value for "titratable acid" may be observed when the ratio to which urinary phosphate has been returned actually differs significantly from that of the glomerular filtrate. This agreement is misleading since the true measure of acid excretion, as has been pointed out, requires restoration of the buffer ratio to that which existed in the filtrate.

It would thus appear that the contribution of phosphate to "titratable acid" can be estimated most accurately by calculation, utilizing the appropriate pK_2' value. However, since pK_2' of phosphate may be influenced by the presence of other electrolytes, a reliable estimate can be made only for urines in which the phosphate salt predominates or in which the effect of some other electrolyte present in high concentration is known. In 24 hour urine specimens, where many electrolytes may be present in high concentration, the situation is more complex and calculation of pK_2' will be less dependable.

If the contribution of phosphate to "titratable acid" is calculated, the contribution of nonphosphate buffers can be determined by titration of dephosphorylated urine. The bulk of organic buffer ordinarily found in urine is comprised of substances whose pK′ values are relatively low in comparison to the pH of plasma. Under these circumstances, no appreciable error will be introduced by ignoring variations in their dissociation constants and in arbitrarily titrating to the plasma pH.

SUMMARY

1. The influence of urinary ionic strength on the apparent dissociation constant of phosphate, pK_2', has been studied during phosphate loading experiments in dogs. pK_2' was determined by measurement of pH at 37° C. after treatment of the urine by a method designed to establish equimolar concentrations of $HPO_4^=$ and $H_2PO_4^-$. The experimental pK_2' values were found to lie between 6.64 and 6.95. Comparison of these pK_2' values with those determined for equimolar sodium phosphate solutions of comparable ionic strength demonstrated agreement within ± 0.02 unit in nearly all instances. The data indicate that, under the present experimental conditions, the behavior of phosphate in urine conformed closely to the predictions of Debye-Hückel theory.

2. The values for pK_2' in the urine as originally excreted were estimated by a method of successive approximations and were found to lie between 6.56 and 6.98.

3. It is concluded that appreciable errors may occur in values of "titratable acid" obtained by calculation or by titration when a pK_2' of 6.8 is arbitrarily assumed for the phosphate system in urine.

ACKNOWLEDGMENTS

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STUDIES IN CUSHING'S SYNDROME. III. URINARY 17-KETOSTEROIDS IN PATIENTS WITH BILATERAL ADRENAL CORTICAL HYPERPLASIA *

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There are several reports in the literature concerning urinary 17-ketosteroids in patients with Cushing's syndrome associated with bilateral adrenal cortical hyperplasia (1–7). In most of the published studies, identification of the individual 17-ketosteroids was incomplete, so that the sum of detailed information available is limited. With the development of improved techniques for steroid fractionation and identification, a re-evaluation of the problem seemed indicated. The present investigation was therefore undertaken with a twofold purpose: *1*) to make a detailed study of the urinary 17-ketosteroids in patients with this form of Cushing's syndrome; and *2*) to determine whether or not the administration of corticotropin to normal subjects was capable of reproducing the excretory pattern of 17-ketosteroids found in Cushing's syndrome with bilateral adrenal cortical hyperplasia. It seemed likely that the data resulting from such studies might provide useful information bearing upon the pathogenesis of this disorder.

MATERIALS AND METHODS

Patients with Cushing's syndrome and bilateral adrenal cortical hyperplasia. Seven patients having the typical clinical and laboratory features of Cushing's syndrome served as the subjects for the study. Pertinent data obtained from these patients are presented in Table I. The absence of adrenal cortical tumor was proved in six of the seven patients by operation (bilateral total or subtotal adrenalectomy). In the seventh patient (Bon.), intravenous pyelography and retroperitoneal insufflation of air gave no evidence of a suprarenal mass. Two of the subjects (Bon. and Fan.) were studied before and during the administration of corticotropin. Bon. received a single intravenous infusion of lyophilized adrenocorticotropic hormone (ACTH), 25 I.U., over an eight hour period. Fan. received corticotropin gel, 40 I.U. intramuscularly twice daily for three days.

Subjects without adrenal cortical disease. Normal values for the individual 17-ketosteroids were derived from a study of 8 normal males (age range, 21 to 35) and 13 normal females (age range, 20 to 31). These subjects were presumably healthy laboratory workers who were carrying on their usual activities. The average values are summarized in Table II. The data are to be published in detail elsewhere.

Two normal females aged 26 and 27 years were studied before and during three day periods of corticotropin administration (ACTH gel, 40 I.U. intramuscularly, twice a day). In addition, studies were carried out in two patients who had been treated with corticotropin for prolonged periods. Patient M.S., a 41 year old white female with bronchial asthma, had received intramuscular doses of 5 to 20 I.U. (lyophilized and gel preparations) with a frequency ranging from once a day to once a week for three years. Patient E.U., a 58 year old white male with rheumatoid arthritis, had been given intramuscular corticotropin (lyophilized and gel) in doses of 2 to 20 I.U. daily or every other day for two years.

Methods. Twenty-four hour urine specimens were collected for periods of one to seven days. If not proc-

TABLE I

Clinical and laboratory data of patients with Cushing's syndrome associated with bilateral adrenal cortical hyperplasia

Name	Age	Sex	"Crude" 17-ks.* (8)	Urine 17-OH-CS†	Plasma 17-OH-CS (17, 42, 43) Before ACTH	Plasma 17-OH-CS (17, 42, 43) After ACTH
	yrs.		*mg./24 hrs.*	*mg./24 hrs.*	*μg./100 ml.*	
Arp.	21	F	23.3	20.3	29	49
Cor.	23	F	11.2	9.2	35	82
Par.	29	M	18.1	11.8	37	61
See.	33	F	26.6	8.5	27	100
Fan.	39	F	42.6	28.8	28	81
Bon.	46	M	22.8	12.5	22	63
Gol.	53	F	19.7	16.6	48	120
Range of normal values			8–20	3–9	4–23	35–55

* 17-Ketosteroids.

† Porter-Silber chromogen after beta-glucuronidase hydrolysis.

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TABLE II

Summary of urinary 17-ketosteroid values in normal subjects, 8 males and 13 females *

		Total identified 17-ks.	Iso.†	Dehydro-iso.	3 beta	% 3 beta	Andro.	Etio.	Etio./ Andro.	3 alpha	% 3 alpha	Total 11-deoxy 17-ks. Amt.	%	11 beta-OH-andro.	11 beta-OH-etio.	11-keto-etio.	*	Total 11-oxy 17-ks. Amt.	%
Males	Average	13.7	1.7	2.7	4.4	31	3.6	3.1	0.9	6.7	50	11.1	81	1.5	0.5	0.6		2.6	19
	Range	5.5–20.5	0.3–3.0	0.1–8.8	0.4–10.0	5–57	1.8–5.7	0.8–6.5	0.43–1.5	2.7–10.9		3.7–14.9	67–91	0.6–3.5	0.2–0.9	0.2–1.2		1.4–5.6	9–33
Females	Average	7.7			1.7‡	22	2.0	2.0	1.0	4.0	52	5.7	74	1.0	0.5	0.5		2.0	26
	Range	4.0–18.0			0.0–6.4‡	0–64	0.9–3.9	0.6–3.8	0.46–2.4	1.9–7.6		3.5–14.0	60–81	0.5–1.7	0.0–1.5	0.0–1.0		1.0–4.0	19–40

* Individual steroid values are given in mg. per 24 hours.

† In this table and Tables III through VI, the following abbreviations for trivial names of steroids are used: iso. for isoandrosterone; dehydroiso. for dehydroisoandrosterone; andro. for androsterone; etio. for etiocholanolone; 11 beta-OH-andro. for 11 beta-hydroxyandrosterone; 11 beta-OH-etio. for 11 beta-hydroxyetiocholanolone; and 11-keto-etio. for 11-ketoetiocholanolone. The systematic names are given in Footnote 1 to the text.

‡ Isoandrosterone and dehydroisoandrosterone were not chromatographically resolved.

TABLE III

Urinary 17-ketosteroids in patients with Cushing's syndrome associated with bilateral adrenal cortical hyperplasia *

Name	Total identified 17-ks.	Iso.†	Dehydro-iso.	3 beta	% 3 beta	Andro.	Etio.	Etio./ Andro.	3 alpha	% 3 alpha	Total 11-deoxy 17-ks. Amt.	%	11 beta-OH-andro.	11 beta-OH-etio.	11-keto-etio.	Total 11-oxy 17-ks. Amt.	%
Arp. ♀	19.9					1.3	8.6	6.6	9.9	50	9.9	50	0.5	3.4	6.1	10.0	50
Cor. ♀	15.7					1.9	3.4	1.8	5.3	34	5.3	34	7.5	2.0	0.8	10.4	66
Par. ♂	17.1					0.9	6.4	7.1	7.3	43	7.3	43	2.9	4.6	2.3	9.8	57
See. ♀	24.7	1.3	2.3	3.6	15	1.6	4.6	2.9	6.2	24	9.8	39	13.0	0.8	1.1	14.9	61
Fan. ♀	49.2		14.0	14.0	28	2.7	12.6	4.7	15.3	32	29.3	60	4.5	8.6	6.8	19.9	40
Bon. ♂	18.9					2.5	5.2	2.1	7.7	41	7.7	41	7.2	1.2	2.8	11.2	59
Gol. ♀	20.7					2.2	7.9	3.6	10.1	49	10.1	49	4.9	3.9	1.8	10.6	51

* Individual steroid values are given in mg. per 24 hours.

† For abbreviations of steroid names see footnote to Table II.

essed immediately, the specimens were refrigerated without preservative.

Total urinary 17-ketosteroids (per 24 hours) were estimated by the colorimetric method of Holtorff and Koch (8). For the isolation of the individual 17-ketosteroids, the urine was subjected to enzymatic hydrolysis (beta-glucuronidase). This was followed by cold, then hot, acid hydrolysis (9). Extracts were chromatographed on alumina according to the technique of Lakshmanan and Lieberman (10). All Zimmermann values were corrected for differences in chromogenicity of the various steroids (11). In every instance, steroids in the chromatographic peaks were identified by infrared spectroscopy.

With this method of fractionation (10) it is not possible to separate chromatographically dehydroisoandrosterone from isoandrosterone, androsterone from Δ^9-androstenolone, or etiocholanolone from Δ^9-etiocholenolone.[1] Quantitation of the individual components of these three binary mixtures was performed by the sulfuric acid chromogen method of Bitman, Rosselet, Reddy and Lieberman (12). Since the Δ^9-unsaturated compounds are probably artifacts of dehydration resulting from hot acid hydrolysis of the 11 beta-hydroxy 17-ketosteroids (13), the quantities of Δ^9-androstenolone and Δ^9-etiocholenolone were added to the values for 11 beta-hydroxyandrosterone and to 11 beta-hydroxyetiocholanolone, respectively. The presence of the Δ^9 steroids and of isoandrosterone was established in each instance by infrared spectroscopy, as well as by the sulfuric acid chromogen method (12).

RESULTS

1. Cushing's syndrome with bilateral adrenal cortical hyperplasia

Values for the individual 17-ketosteroids in the seven patients with bilateral adrenal cortical hyperplasia are shown in Table III. The total quantity of identified 17-ketosteroids was moderately increased in three subjects (Arp., See., Gol.), markedly increased in one (Fan.), and normal in the remaining three (Cor., Par. and Bon.).

Analysis of urinary 17-ketosteroids revealed three findings of note. *1*) When compared with the normal subjects (Table II), all seven patients with Cushing's syndrome were observed to excrete excessive amounts of 11-oxygenated 17-ketosteroids. The proportion of 11-oxygenated 17-ketosteroids relative to total ketosteroids was also greater than normal. 11 Beta-hydroxyandrosterone, 11 beta-hydroxyetiocholanolone, and 11-ketoetiocholanolone together comprised 40 to 66 per cent of the total as compared with 9 to 40 per cent in the normal individuals (Tables II and III). Of special interest was the relatively large amount of 11 beta-hydroxyandrosterone identified in the urine of five of the seven patients (all except Arp. and Par., Table III) (see Discussion). *2*) In six of the seven patients (all except Fan.), the total quantity of 11-deoxy 17-ketosteroids was within normal limits (compare data of Tables II and III). A striking finding in five of the seven (all except Cor. and Bon.) was the alteration in the ratio of etiocholanolone to androsterone. In the normal subject, the ratio of the two steroids averages about 1 (Table II). The average etiocholanolone to androsterone ratio in these patients with Cushing's syndrome was 4.1 (Table III). *3*) In only two of the seven patients with Cushing's syndrome was there a detectable quantity of the beta ketosteroids, isoandrosterone and dehydroisoandrosterone (Patients See. and Fan., Table III). Neither of these two subjects excreted an excessive proportion of beta ketosteroids, but Fan. showed a marked increase in the absolute quantity of dehydroisoandrosterone (14.0 mg. per 24 hours as compared with 6.4 mg. per 24 hours, the extreme upper limit observed in the normal females).

Effects of corticotropin administration. Table IV shows the urinary excretion values for the 17-ketosteroids following corticotropin administration to Patients Bon. and Fan. A greater rise in 11-oxygenated than in 11-deoxy 17-ketosteroids was observed. The ratio of etiocholanolone to androsterone was not increased over the control value in either patient. Both of these findings are similar to those reported by Kappas, Dobriner

[1] The following trivial names for C19 steroids are used in this paper: androsterone for 3α-hydroxyandrostan-17-one; etiocholanolone for 3α-hydroxyetiocholan-17-one; dehydroisoandrosterone for 3β-hydroxy-Δ^5-androsten-17-one; isoandrosterone for 3β-hydroxyandrostan-17-one; Δ^9-androstenolone for 3α-hydroxy-$\Delta^{9,11}$-androsten-17-one; Δ^9-etiocholenolone for 3α-hydroxy-$\Delta^{9,11}$-etiocholen-17-one; 11β-hydroxyetiocholanolone for 3α,11β-dihydroxyetiocholan-17-one; 11β-hydroxyandrosterone for 3α,11β-dihydroxyandrostan-17-one; 11-ketoetiocholanolone for 3α-hydroxyetiocholane-11,17-dione; and 11-ketoandrosterone for 3α-hydroxyandrostane-11,17-dione. For certain C21 steroids, the following trivial names are employed: hydrocortisone for 11β,17α,21-trihydroxy-Δ^4-pregnene-3,20-dione; Substance S for 17α,21-dihydroxy-Δ^4-pregnene-3,20-dione; tetrahydro E for 3α,17α,21-trihydroxypregnane-11,20-dione; tetrahydro F for 3α,11β,17α,21-tetrahydroxypregnan-20-one; tetrahydro S for 3α,17α,21-trihydroxypregnan-20-one; 17α-hydroxyprogesterone for 17α-hydroxy-Δ^4-pregnene-3,20-dione; 17α-hydroxypregnanolone for 3α,17α-dihydroxypregnan-20-one; and pregnanetriol for pregnane-3α,17α,20α-triol.

TABLE IV

Effect of corticotropin administration on urinary 17-ketosteroid excretion in two patients with Cushing's syndrome and bilateral adrenal hyperplasia *

Subject		Total	Iso.†	Dehydro-iso.	3 beta	% 3 beta	Andro.	Etio.	Etio./ Andro.	3 alpha	% 3 alpha	Total 11-deoxy 17-ks. Amt.	%	11 beta-OH-andro.	11 beta-OH-etio.	11-keto-etio.	Total 11-oxy 17-ks. Amt.	%
Bon.	Before ACTH	9.7					1.9	2.9	1.5	4.8	50	4.8	50	3.3	0.9	0.7	4.9	50
	After ACTH	15.8					3.7	3.8	1.0	7.5	47	7.5	47	6.7	1.4	0.2	8.3	53
Fan.	Before ACTH	49.2		14.0	14.0	28	2.7	12.6	4.7	15.3	32	29.3	60	4.5	8.0	6.8	19.9	40
	After ACTH	76.5		19.7	19.7	26	6.7	16.4	3.5	21.1	27	40.8	53	11.0	19.0	5.7	35.7	47

* Values for steroids are given in mg. per 24 hours.
† For abbreviations of steroid names, see footnote to Table II.

TABLE V

Effect of short-term administration (three days) upon urinary 17-ketosteroid excretion in two normal women *

Subject		Total	Iso.†	Dehydro-iso.	3 beta	% 3 beta	Andro.	Etio.	Etio./ Andro.	3 alpha	% 3 alpha	Total 11-deoxy 17-ks. Amt.	%	11 beta-OH-andro.	11 beta-OH-etio.	11-keto-etio.	Total 11-oxy 17-ks. Amt.	%
M. W.	Before ACTH	6.3	0.6	0.3	0.9	14	1.5	2.2	1.5	3.7	59	4.6	73	0.7	0	1.0	1.7	27
	After ACTH	18.8	0.8	1.3	2.1	11	4.1	4.5	1.1	8.6	46	10.7	57	4.1	2.0	2.0	8.1	43
E. E.	Before ACTH	4.9					1.4	1.7	1.2	3.1	63	3.1	63	0	0.3	1.5	1.8	37
	After ACTH	12.3					1.8	3.0	1.7	4.8	39	4.8	39	0.5	3.9	3.1	7.5	61

* Steroid values are given in mg. per 24 hours.
† For abbreviations of steroid names, see footnote to Table II.

and Gallagher for normal subjects to whom ACTH was administered (14, 15).

2. Effects of corticotropin in subjects without adrenal cortical disease

Table V shows the excretion values for the 17-ketosteroids before and during the intramuscular administration of ACTH to two normal women over a three day period. In both subjects, there was the expected increase in excretion of 11-oxygenated 17-ketosteroids, the increase being relatively greater than that observed for the 11-deoxy-17-ketosteroids. In M.W., 11 beta-hydroxyandrosterone excretion increased more than did the excretion of 11 beta-hydroxyetiocholanolone or 11-keto etiocholanolone. No significant change in the ratio of etiocholanolone to androsterone was observed in either subject over this short period of ACTH administration, a finding also reported by Kappas and Gallagher for normal women treated with ACTH (15).

The effects of long-term ACTH administration upon 17-ketosteroid excretion are presented in Table VI. Patient M.S. had an abnormal excretory pattern of 17-ketosteroids with a relative increase in the quantity of 11-oxygenated 17-ketosteroids. Only a trace of androsterone could be detected, and 1.4 mg. of etiocholanolone was present. In E.U., the ratio of etiocholanolone to androsterone was approximately 10 (compare Table II, normal subjects). The quantity of the individual 11-oxygenated 17-ketosteroids could not be exactly determined since 11 beta-hydroxyandrosterone was not chromatographically separated from 11-ketoetiocholanolone.

DISCUSSION

The urinary 17-ketosteroid pattern observed in these patients with Cushing's syndrome and bilateral adrenal cortical hyperplasia is a characteristic one, and differs from the ketosteroid pattern found in normal subjects and from that reported in other types of adrenal cortical disease (16, 30, 32). But the abnormal 17-ketosteroid pattern in Cushing's syndrome with adrenal hyperplasia derives its chief interest from its possible bearing upon the problem of deranged adrenal cortical secretion, and further, from its possible bearing upon the pathogenesis of the disorder. In the following discussion, it is assumed that the urinary steroid pattern is a more or less direct reflection of adrenal cortical secretion. The possibility cannot be excluded that some or all of the abnormalities observed might have been related to changes in the peripheral metabolism of adrenal steroids, changes which are peculiar to Cushing's syndrome. To date, no such metabolic errors in Cushing's syndrome have been demonstrated.

The increase in the urinary excretion of 11-oxygenated 17-ketosteroids is not surprising and can be adequately accounted for by the increased amount of hydrocortisone which is presumably secreted by the adrenal cortex in Cushing's syndrome. The increase in hydrocortisone secretion appears to be amply documented by the findings of elevated plasma hydrocortisone levels (17–19) and increased urinary excretion of C21 metabolic products of hydrocortisone, tetrahydro E and tetrahydro F (20). 11 Beta-hydroxyandrosterone, 11 beta-hydroxyetiocholanolone, and 11-ketoetiocholanolone have been shown to be the principal

TABLE VI

Urinary 17-ketosteroids after long-term corticotropin (two and three years) *

Subject	Total	3 beta	Andro.†	Etio.	Etio./ Andro.	3 alpha	% 3 alpha	Total 11-deoxy 17-ks. Amt.	%	11β-OH andro.	11β-OH etio.	11-keto-etio.	Total 11-oxy 17-ks. Amt.	%
M. S.	5.9	0	tr	1.4	>1.4	1.4	24	1.4	24	1.3	2.0	1.2	4.5	76
E. U.	9.4	0	0.5	5.3	10.6	5.8	62	5.8	62	‡		‡	3.6‡	38

* Values for steroids are given in mg. per 24 hours.
† For abbreviations of steroid names, see footnote to Table II.
‡ 11-Ketoetiocholanolone and 11 beta-OH-androsterone were not chromatographically resolved.

C19 excretory products of administered hydrocortisone [2] (22).

The relative proportions of the individual 11-oxy 17-ketosteroids one to another are not readily accounted for by an increased secretion of hydrocortisone alone. The infusion of 4-C^{14}-labeled hydrocortisone in man has been reported to result in the urinary excretion of labeled 11 beta-hydroxyandrosterone, 11 beta-hydroxyetiocholanolone, and 11-ketoetiocholanolone in the ratio 2:9:9 (21). The ratio of the three steroids observed in certain of the patients with Cushing's syndrome is very different from this (Table III, Patients Cor., See., Bon. and Gol., in whom these ratios were approximately 8:2:1, 13:1:1, 7:1:3 and 5:4:2). It is possible that a precursor other than hydrocortisone may contribute in an important way to the excretion of 11-oxygenated 17-ketosteroids in Cushing's syndrome. One such precursor might conceivably be Δ^4-androstene-11 beta-ol-3,17-dione, a normal constituent of adrenal vein blood, which has been shown to be converted to 11 beta-hydroxyandrosterone, 11 beta-hydroxyetiocholanolone, and 11-ketoetiocholanolone in the ratio 12:1:2 (23).

The ratio of etiocholanolone to androsterone in five of the seven patients with Cushing's syndrome is above the extreme upper limit found in normal individuals in the present investigation (compare Tables II and III) and in earlier studies by Kappas, Dobriner and Gallagher [3] (14, 15). The average ratio of etiocholanolone to androsterone in normal subjects is close to unity (Table II) (14, 15). The identity of the adrenal cortical precursor of etiocholanolone and androsterone in normal subjects is not known, but the 1:1 urinary ratio of the two steroids has led to speculation concerning their origin. For example, it has been postulated (14, 15, 25–27) that the precursor is a C19 11-deoxy steroid because, in a limited number of studies, it has been shown that the administration of certain of these steroids (testosterone, dehydroisoandrosterone, Δ^4-androstene-3,17-dione) results in the urinary excretion of etiocholanolone and androsterone in roughly equal proportions [etiocholanolone: androsterone 1.5:1 (26), average etiocholanolone: androsterone ratio in several experiments 1.7:1 (27)]. Thus, although a C19 precursor may contribute to the quantity of etiocholanolone in the patients with Cushing's syndrome, a major portion of this steroid may well be derived from a C21 precursor. One precursor which might be supposed to account for the altered ratio of etiocholanolone to androsterone is the C21 11-deoxy 17-hydroxysteroid, Reichstein's Substance S. Administration of this steroid to human subjects has been found to result in the urinary excretion of greater quantities of etiocholanolone than of androsterone (28, 29). Another 11-deoxy 17-hydroxy steroid of the C21 series, 17 alpha-hydroxyprogesterone, administration of which has been shown to give rise to a high etiocholanolone: androsterone ratio, does not appear to be a likely precursor because its principal excretory products, pregnanetriol and 17 alpha-hydroxypregnanolone, are not increased in this form of Cushing's syndrome (30). In a few of these patients, pregnanetriol was found in amounts not exceeding normal. In any event, it would seem that a search for the 11-deoxy 17-ketosteroid precursors in this disorder should be directed toward C21 rather than C19 compounds (27).

Dehydroisoandrosterone was not isolated in excess from any patient with Cushing's syndrome except Fan. (Table III). This observation is in accord with the results of most studies of urinary 17-ketosteroids in Cushing's syndrome (31). It has been found by most investigators that excessive quantities of dehydroisoandrosterone are not present in the urine of patients with adrenal hyperplasia, but that large amounts of this steroid may be present in the urine of patients with adrenal cortical carcinoma (31). Recent studies suggest that abnormally large quantities of dehydroisoandrosterone are by no means invariably found in adrenal cortical carcinoma (32). Be-

[2] Gallagher was unable to show that hydrocortisone administration gives rise to an appreciable quantity of urinary 11-keto-androsterone (21). In the present study, it was also impossible to identify this steroid conclusively in any patient. With the methods used, trace amounts (less than 100 μg. per 24 hours) could have gone undetected.

[3] The high average ratio of etiocholanolone (4:1) cannot be explained on the basis of excessive adrenal secretion of hydrocortisone. Studies of urinary 17-ketosteroids after cortisone or hydrocortisone administration have not shown increased urinary excretion of any 11-deoxy 17-ketosteroid (14, 22). In fact, there has been to date no demonstration of the *in vivo* removal of the oxygen function at C11 from the steroid nucleus (24).

cause the precursor (or precursors) of dehydroisoandrosterone is unknown at this time, the true significance of this steroid as a urinary excretory product is difficult to assess (33, 34).

Since all of the clinical features of Cushing's syndrome can be reproduced by the administration of ACTH, it was considered important to determine whether or not this pituitary hormone was capable of bringing about the changes in urinary 17-ketosteroid excretion which appeared to be characteristic of the naturally-occurring disorder. The present studies are in agreement with previous work in that they show a greater relative rise in 11-oxy than in 11-deoxy 17-ketosteroid excretion after the short-term administration of ACTH (15). The failure to bring about an increase in the ratio of etiocholanolone to androsterone with a short course of ACTH is also in agreement with earlier work (14, 15). In contrast, the administration of corticotropin to two patients for two and three years was associated with an increase in this ratio, which, in Patient E.U. (Table VI), was quantitatively comparable to the change in etiocholanolone: androsterone ratio observed in the patients with adrenal cortical hyperplasia (Table III). On the basis of the limited evidence now available, it seems improbable that chronic disease alone (*e.g.*, rheumatoid arthritis as in E.U.) satisfactorily accounts for the abnormality in 11-deoxy 17-ketosteroid excretion. Birke has reported that the ratio of etiocholanolone to androsterone in a group of patients with long-standing rheumatoid arthritis was essentially normal (35).

Concerning possible alterations in 17-ketosteroid precursors secreted by the ACTH-stimulated adrenal cortex, the observations of Touchstone, Bulaschenko, Richardson and Dohan may be pertinent (20). These workers have reported the isolation of a metabolic product of Substance S, tetrahydro S, from the urine of ACTH-treated subjects. Although corticotropin-treated normals and patients with Cushing's syndrome associated with bilateral adrenal hyperplasia have high etiocholanolone: androsterone ratios (present study), one hesitates to draw an analogy between the two groups. This is because tetrahydro S has to date been definitely identified only in the urine of patients with Cushing's syndrome associated with adrenal cortical carcinoma (20, 36), and only tentatively identified in urine of patients with adrenal hyperplasia (20). It is also necessary to exercise caution in equating bilateral adrenal cortical hyperplasia with ACTH-treated normal subjects, since several investigators have failed to demonstrate increased quantities of circulating ACTH in patients with Cushing's syndrome (37, 38), although abnormal corticotropic activity of unknown origin has been reported (39).

It is conceivable that administered ACTH, given over long periods, may be capable of causing profound qualitative changes in corticosteroid biosynthesis, perhaps by affecting the relative activities of the various steroid hydroxylases in the adrenal cortex [*e.g.*, 11 beta-hydroxylase (40)]. An example of such a change may perhaps be found in the observation of Kass, Hechter, Macchi and Mou that prolonged ACTH administration altered the ratio of hydrocortisone to corticosterone in the adrenal vein blood of rabbits (41). A possible relationship between the corticotropin-stimulated adrenal cortex and naturally-occurring adrenal cortical hyperplasia remains a subject for future investigation.

SUMMARY

In Cushing's syndrome associated with bilateral adrenal cortical hyperplasia, urinary 17-ketosteroids were fractionated by means of column chromatography and identified by infrared spectroscopy. The excretory pattern of 17-ketosteroids in seven patients with this disorder exhibited the following characteristics:

1. An absolute and relative increase in the 11-oxygenated 17-ketosteroids.

2. An absolute increase in the quantity of 11 beta-OH-androsterone which was out of proportion to the value that would be expected on the basis of excessive adrenal secretion of hydrocortisone alone.

3. An average ratio of etiocholanolone to androsterone approximately four times greater than that observed in normal subjects. Total values of androsterone were normal in all seven; total values of etiocholanolone were above the upper limit of normal in four.

4. The absence of detectable quantities of dehydroisoandrosterone in five of the seven patients;

the presence of an abnormally large quantity of dehydroisoandrosterone in one of the seven.

Prolonged administration of corticotropin to two patients without adrenal cortical disease was associated with a high ratio of etiocholanolone to androsterone.

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THE EFFECT OF THE ADMINISTRATION OF SODIUM BICARBONATE AND AMMONIUM CHLORIDE ON THE EXCRETION AND PRODUCTION OF AMMONIA. THE ABSENCE OF ALTERATIONS IN THE ACTIVITY OF RENAL AMMONIA-PRODUCING ENZYMES IN THE DOG

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The regulation of the excretion of ammonia by the kidney has been studied extensively. It is clear that urine pH is an important determinant of ammonia excretion, with greater amounts of ammonia diffusing into acid urines than into alkaline urines (1–8). However, it is also evident that the magnitude of the rise in ammonia excretion during the chronic administration of strong acids or acidifying salts cannot be accounted for solely on the basis of "trapping" of greater amounts of ammonia in increasingly acid urines. Thus Pitts (4) found that dogs given NH_4Cl for two days excreted more ammonia at any given urine pH than did control animals, presumably due to increased production of ammonia by the renal tubule cells. Earlier, Van Slyke and co-workers (9) had found that the extraction of glutamine from renal arterial blood was greater in dogs receiving NH_4Cl than in those receiving $NaHCO_3$, and further that the extracted glutamine amide-N accounted for approximately 60 per cent of the ammonia produced by the kidney. Lotspeich and Pitts (10) postulated that the capacity of some renal mechanism, capable of extracting and hydrolyzing glutamine, increases during chronic metabolic acidosis to produce more ammonia. This was confirmed in rats when Davies and Yudkin (11) and others (7, 12) found that the administration of HCl increased and the administration of $NaHCO_3$ decreased the activity of rat kidney glutaminase. The observation of similar changes in acidotic guinea pigs and rabbits (13) has suggested that ammonia excretion in all mammals, including man, is regulated in part by adaptive changes in the activity of renal glutaminase.

There is evidence, however, that the regulation of ammonia excretion differs in dogs and rats. Orloff and Berliner (6) observed that the relationship between urine pH and ammonia excretion in dogs was reproducible following the infusion of sodium bicarbonate and independent of acute alterations in acid-base balance. This is in contrast to the findings of Leonard and Orloff (7) in the rat. In this species a correlation between ammonia excretion and urine pH was not observed under all circumstances. Alterations in ammonia excretion occurred independently of urine pH changes in some circumstances. The converse was also observed: changes in urine pH without associated changes in ammonia excretion. In the rat variations in ammonia excretion in acute studies are conditioned not only by urine pH, the only determinant in the dog, but also by the state of acid-base balance. Dogs and rats also exhibit dissimilar urinary responses to the chronic inhibition of carbonic anhydrase. In rats treated chronically with acetazoleamide, ammonia excretion falls briefly, then rises to greater than normal levels. This elevated excretion of ammonia is associated with an increased activity of renal glutaminase (7, 14). In dogs, however, the chronic administration of acetazoleamide results in a persistent reduction in ammonia excretion, with a return towards normal values only as the urine becomes less alkaline (15). On the basis of the response of man (16) and dog (15) to acetazoleamide, it had been suggested (17) that an adaptive increase in the activity of renal glutaminase might not occur in these species.

The purpose of the present studies was to determine whether or not alterations in the activity of renal glutaminase participate in the regulation of ammonia excretion in dogs. The effects of NH_4Cl and $NaHCO_3$ on the relationship between

ammonia excretion and the activities of the various renal enzymes involved in the conversion of glutamine to ammonia were examined in dogs and the results compared with those from essentially analogous studies in rats.

PROCEDURES

The relationship between ammonia excretion and urine pH in four trained female dogs was determined both after the administration of 8 Gm. of NH_4Cl daily for 7 to 14 days and after the administration of an equivalent amount of $NaHCO_3$ for a similar period of time. At the end of each experimental period studies were performed in which the urine was acutely acidified by the infusion of 200 μM per minute of Na_2SO_4, then alkalinized by the infusion of 600 μM per minute of $NaHCO_3$, using a Bowman constant infusion pump. Urine was collected anaerobically in oiled syringes for pH and ammonia measurements.

To study the effect of NH_4Cl and $NaHCO_3$ on the activities of the renal enzymes involved in the production of ammonia, biopsies of kidney cortex were obtained surgically from 12 dogs and the activities of the enzymes compared after the administration of NH_4Cl and after the administration of $NaHCO_3$. Thus each dog served as its own control. Enzymatic activity was also determined in homogenates of kidneys from both acidotic and normal rats. Six of the 12 dogs were given 8 Gm. of $NaHCO_3$ daily for 5 to 21 days, after which time the kidney biopsies and enzyme analyses were performed. The dogs were then given 8 Gm. NH_4Cl daily for 5 to 21 days and the kidney enzyme determinations repeated. In the remaining 6 dogs the procedure was reversed so that NH_4Cl was given first and $NaHCO_3$ last. Four of the dogs received 100 mEq. of HCl intravenously daily instead of the oral NH_4Cl. This was done in order to circumvent any possible difficulties due to inadequate absorption. At the time of biopsies blood samples were drawn for plasma bicarbonate measurements. Immediately upon removal, the samples of kidney cortex were either weighed and homogenized in 19 volumes of ice cold distilled water to give 5 per cent homogenates, or sliced in the cold with a Stadie-Riggs slicer. The homogenates were assayed for phosphate-activated glutaminase I, pyruvate-activated glutaminase II, glutamic dehydrogenase and glutamic-oxaloacetic transaminase.

METHODS

The pH of the urine was measured in an internal glass electrode at 37° C. using a Cambridge research model pH meter. The concentration of ammonia in the urine was measured by the method of Conway (18). The total CO_2 content of plasma was determined by the method of Van Slyke and Neill (19). Dry weights of the kidney homogenates were determined by drying 5 ml. aliquots of the homogenates overnight at 90° C.

The activity of phosphate-activated glutaminase I was assayed using the method previously described (14). The pH optimum (8.0) of glutaminase I was the same in the kidneys of rats and dogs, and the rate of ammonia formation was linear over the same range of tissue concentrations (5 to 20 mg. wet kidney) for both dog and rat kidneys.

The activity of pyruvate-activated glutaminase II was measured by a modification of the method described by Goldstein, Richterich-van Baerle and Dearborn (20). Maximal activity for dog and rat kidney glutaminase II was observed at pH 8.8 to 9.0. The method was modified by substituting 0.5 M Tris buffer (pH 9.0) for the veronal buffer. At the end of the 30 minute incubation period the reaction was stopped by the addition of 0.2 ml. 37.5 per cent $HClO_4$. The samples were centrifuged and ammonia measured on 0.2 ml. aliquots of the supernatant by a modification of the microdiffusion method of Seligson and Seligson (21). Rather than using the Nesslers reagent as described by Seligson the ammonia was determined colorimetrically using hypo-chlorous acid and sodium phenate (22).

Glutamine synthetase activity was measured in several different ways: the uptake of ammonia by kidney homogenate in the presence of glutamate and ATP as described by Richterich-van Baerle and associates (23), the formation of γ-glutamyl hydroxamate as described by Levintow, Meister, Hogeboom and Kuff (24), and the method of Reiner and Hudson (25). Potent activity was demonstrable in rat kidney using all three methods, but no measurable activity was found in dog kidney with any of the methods. Since Levintow and co-workers (24) have adduced evidence that the same enzyme catalyzes both the synthesis of glutamine and the transfer exchange of the γ-glutamyl amide group, the formation of γ-glutamyl hydroxamate from glutamine, ADP, and hydroxylamine as described by Levintow and associates (24) was the method finally adopted for the assay of glutamine synthetase activity in rat kidney homogenates. Five-tenths ml. of a 1 per cent homogenate was used in each assay. γ-Glutamyl hydroxamate was measured by the method of Lipmann and Tuttle (26).

Glutamic dehydrogenase activity was assayed in 0.2 ml. of a 1 : 6 dilution of the 5 per cent homogenate using the method of Olson and Anfinsen (27). Glutamic-oxaloacetic transaminase was assayed in 0.2 ml. of a 1 : 25 dilution of the 5 per cent kidney homogenate according to the method of Steinberg and Ostrow (28).

The hydrolysis of glutamine by intact kidney slices was determined by incubating slices of kidney cortex, weighing 50 to 100 mg., in 3 ml. of oxygenated Ringer's phosphate solution (pH 7.4), containing 49 μM glutamine per ml. The reaction was stopped at the end of 30 minutes by the addition of 0.2 ml. 37.5 per cent $HClO_4$. The medium was centrifuged and ammonia was measured in 0.5 ml. aliquots of the supernatant.

RESULTS

The effect of the chronic administration of NH_4Cl and $NaHCO_3$ on the relationship between

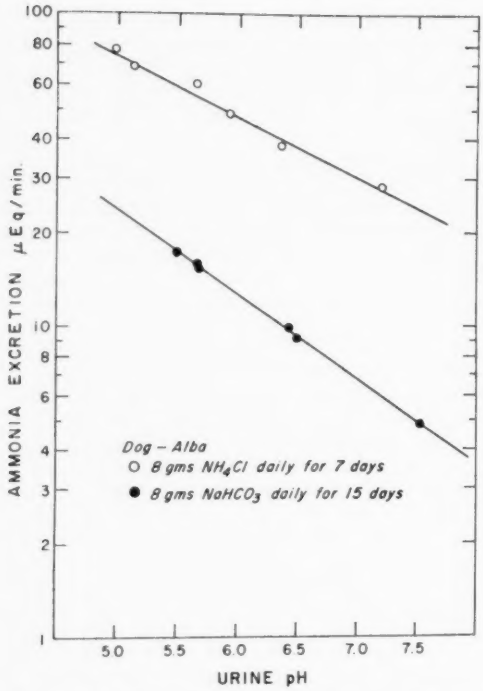

FIG. 1. THE EFFECT OF CHRONIC NH_4Cl AND $NaHCO_3$ LOADS ON THE RELATION BETWEEN AMMONIA EXCRETION AND URINE pH IN DOGS

ammonia excretion and urine pH is shown in Figure 1. In both studies a linear relationship between the logarithm of ammonia excretion and urine pH was observed. Although the slopes of the two lines are approximately the same, the rate of ammonia excretion is three to four times greater at any given urine pH after NH_4Cl than after $NaHCO_3$. The results from three other dogs were similar. Adaptive changes in ammonia excretion have also been reported in rats (7) and guinea pigs (29). The results of these studies confirm the observations of Pitts (4) that increased excretion of ammonia after the chronic administration of NH_4Cl cannot be accounted for on the basis of increased acidity of the urine, but must in some way reflect accelerated production of ammonia by renal tubule cells.

As noted earlier, the studies of Van Slyke and associates (9) indicate that the increased production of ammonia during NH_4Cl acidosis is a consequence of enhanced hydrolysis of glutamine. In rats (7, 11, 12) and guinea pigs (13) this accelerated conversion of glutamine to ammonia is apparently accomplished by an adaptive increase in the activity of renal glutaminase. The results of the present studies, however, indicate that such a mechanism does not account for the increased ammonia excretion in acidotic dogs.

The effect of the chronic administration of NH_4Cl and $NaHCO_3$ on the renal glutaminases is shown in Table I. In rats NH_4Cl increased the activity of phosphate-activated glutaminase I approximately fourfold, whereas in dogs NH_4Cl resulted in a 10 per cent decrease in activity. A similar decrease was noted in four dogs given HCl intravenously rather than NH_4Cl orally. Although this decrease in the activity of dog kidney glutaminase I was statistically significant, its physiological significance is obscure. Pyruvate-activated glutaminase II was also increased approximately fourfold in rats given NH_4Cl, but was unchanged in dogs. Glutamine synthetase activity, estimated indirectly from γ-glutamyl transferase activity, was decreased in rats receiving NH_4Cl, while no activity was measurable in dog kidneys. The depression of glutamine synthetase activity in rat kidneys is in contrast to the results of Richterich-van Baerle and coworkers in which glutamine synthetase activity was increased in guinea pig kidneys by the administration of NH_4Cl. It is unlikely that the failure of glutaminase I and II to increase in dogs during the administration of NH_4Cl was due to inadequate stimulation, since at the time of biopsy the plasma HCO_3^- concentrations were 26 ± 2 mEq. per L. after $NaHCO_3$ and 21 ± 2 mEq. per L. after NH_4Cl, and the ammonia excretions were 8 mEq. per 24 hours after $NaHCO_3$ and 45 mEq. per 24 hours after NH_4Cl. Thus it is seen that the dogs were in fact slightly acidotic and were excreting increased amounts of ammonia after the administration of NH_4Cl.

Since the administration of NH_4Cl did not increase the activity of glutaminase I and II in the cortex of dog kidneys, the possibility of isolated changes occurring in other sections of the kidney was investigated. The activity of glutaminase I was measured in the cortex, medulla and papilla of kidney from dogs given either NH_4Cl or $NaHCO_3$ and in rats given either H_2O or 1.5 per cent NH_4Cl solution *ad lib.* In both dogs and

TABLE I

The effect of NH_4Cl and $NaHCO_3$ on the activity of glutaminase I, glutaminase II and glutamine synthetase in kidney cortex of dogs and rats

Group		Phosphate-activated glutaminase *μM NH_3/hr./100 mg. dry kidney*		Pyruvate-activated glutaminase *μM NH_3/hr./100 mg. dry kidney*		Glutamine synthetase *units/hr./100 mg. wet kidney*	
		Dogs	Rats	Dogs	Rats	Dogs	Rats
No. of animals		12	8	4	4	4	4
Controls	Mean activity		545		101		45.1
	S. D.		±77		±31		±3.9
$NaHCO_3$*	Mean activity	735		105			
	S. D.	±151		±15			
NH_4Cl†	Mean activity	665	2,120	108	471	0	38.0
	S. D.	±175	±412	±53	±146		±2.9
	Mean difference	−70	+1,595	+ 3	+370		−7.1
	S. E.	±30	±148	±22	± 57		±2.5
	p	<0.05	<0.01	>0.5	<0.01		<0.05

* The dogs received 8 Gm. $NaHCO_3$ daily for 5 to 21 days.
† The dogs received 8 Gm. NH_4Cl daily for 5 to 21 days; the rats received 1.5 per cent NH_4Cl in tap water *ad lib.* for 14 to 21 days.

rats the activity of glutaminase I in the papilla was approximately one-tenth the activity in the cortex. In rats the administration of NH_4Cl had no effect on the activity of glutaminase I in the papilla, while the activity increased approximately fourfold in the cortex. In dogs the administration of NH_4Cl did not increase the activity of glutaminase I in either cortex or medulla. A slight increase in activity was observed in the papilla of dogs. However the enzyme and tissue blank constituted such a large fraction of the total ammonia produced that the results were not interpretable. Furthermore the absolute amounts of ammonia produced were so small as to be physiologically insignificant.

Although the activity of the renal glutaminases, as assayed in homogenates, did not increase in dogs given NH_4Cl, the alternative possibility that chronic NH_4Cl acidosis induces changes in the intracellular environment which increases the effective activity of the enzyme resident in the cell, without changing its actual concentration, was considered. Changes of such a nature would not be detected by assays of tissue homogenates, but might be with intact kidney slices. To investigate this point the rate of glutamine hydrolysis by intact kidney slices was measured. The effect of NH_4Cl administration is shown in Table II. NH_4Cl increased the rate of hydrolysis in rats, but decreased the rate by approximately 10 per cent in dogs. Although no evidence for an increase in the effective intracellular activity of dog kidney glutaminase was obtained, the true *in vivo* activity may not be reflected by the *in vitro* hydrolysis of glutamine by kidney slices.

Another process whereby the conversion of glutamine to ammonia could be accelerated involves the action of glutamic dehydrogenase. Glutamic

TABLE II

The effect of NH_4Cl and $NaHCO_3$ on the hydrolysis of glutamine by intact kidney slices

Group		Glutamine hydrolysis *μM NH_3/100 mg. kidney/hr.*	
		Dogs	Rats*
No. of animals		4	12
$NaHCO_3$†	Mean activity	7.7	5.5
	S. D.	±0.7	±0.8
NH_4Cl‡	Mean activity	7.2	14.0
	S. D.	±1.0	±2.3
	Mean difference	−0.45	+8.4
	S. E.	±0.36	±0.7
	p	>0.30	<0.01

* Unpublished data of J. Orloff.
† The dogs received 8 Gm. $NaHCO_3$ daily for 10 to 14 days; the rats received 1.5 per cent $NaHCO_3$ solution *ad lib.* for 14 days.
‡ The dogs received 8 Gm. NH_4Cl daily for 10 to 14 days; the rats received 1.5 per cent NH_4Cl solution *ad lib.* for 14 days.

TABLE III

The effect of NH_4Cl and $NaHCO_3$ on glutamic dehydrogenase and glutamic-oxaloacetic transaminase in kidney cortex of dogs and rats

Group		Glutamic acid dehydrogenase *units/mg. dry kidney*		Glutamic-oxaloacetic transaminase *units/mg. dry kidney*	
		Dogs	Rats	Dogs	Rats
No. of animals		12	4	5	4
Controls	Mean activity		380		489
	S. D.		± 59		± 34
$NaHCO_3$*	Mean activity	326		285	
	S. D.	±80		±57	
NH_4Cl†	Mean activity	278	955	281	485
	S. D.	±92	±117	±32	±126
	Mean difference	−48	+575	− 4	− 4
	S. E.	±32	± 66	±18	± 65
	p	<0.2	<0.01	>0.5	>0.5

* Dogs received 8 Gm. $NaHCO_3$ daily for 5 to 21 days.
† Dogs received 8 Gm. NH_4Cl daily for 5 to 21 days; rats received 1.5 per cent NH_4Cl *ad lib.* for 14 to 21 days.

acid, an end product of glutamine hydrolysis, is known to inhibit glutaminase I (30). An increase in the activity of glutamic dehydrogenase, by increasing the rate of glutamic acid removal, would increase the effective activity of glutaminase I. Also the action of glutamic dehydrogenase would permit glutamine to contribute its amino-N as well as its amide-N to urinary ammonia. As seen in Table II the administration of NH_4Cl increased the activity of glutamic dehydrogenase by approximately threefold in rats, but decreased the activity slightly in dogs.

The participation of ammonia precursors other than glutamine in the increased formation of ammonia during NH_4Cl acidosis was also investigated. Davies and Yudkin (11) found that in addition to renal glutaminase, L-amino acid oxidase and glycine oxidase were increased in the kidneys of rats given HCl. Although, no L-amino acid oxidase has been found in dog kidney (31), infusions of amino acids are known to stimulate ammonia excretion in dogs (6, 10, 32). The probable pathway involved is transamination of the amino acid with γ-keto glutarate to form glutamate, which is then deaminated. The possibility that this pathway was responsible for the increased production of ammonia in acidotic dogs was studied. However, the administration of NH_4Cl produced no changes in the activity of glutamic-oxaloacetic transaminase in either dogs or rats (Table III).

DISCUSSION

Recent investigations of the physicochemical factors influencing ammonia excretion (6, 8, 33) support the hypothesis that ammonia transverses the tubule cell membrane as the unionized lipoid soluble NH_3, and that, except for very acid urines, equilibrium between cell and luminal NH_3 is attained. Since the NH_4^+ ion appears to be relatively nondiffusible across the cell membrane and is in rapid equilibrium with NH_3, the accumulation of NH_4^+ in the urine is dependent on urine pH, more accumulating in acid than in alkaline urines. In addition, any factor which changes the concentration of ammonia within the cell alters ammonia excretion more or less independently of urine pH. Thus, infusions of glutamine and other amino acids elevate ammonia excretion despite alkalinization of the urine (6, 32).

During the chronic administration of NH_4Cl cellular factors appear to be more important in regulating ammonia excretion than is urine pH. Animals receiving NH_4Cl excrete more ammonia at any given urine pH than do control animals, indicating that the concentration of ammonia in the tubule cell has risen, presumably due to increased production of ammonia.

The increased production of ammonia during NH_4Cl acidosis appears to be related primarily to the hydrolysis of glutamine (9), and to a lesser extent to the deamination of other amino acids

(9). In rats elevation of the activity of the renal ammonia-producing enzymes appears to be responsible for the accelerated production of ammonia (7, 11, 12). The administration of NH_4Cl produces striking increases in the activity of glutaminase I, glutaminase II, glutamic dehydrogenase, L-amino acid oxidase and glycine oxidase. In dogs, however, adaptation of the renal ammonia-producing enzymes does not participate in the regulation of ammonia excretion. In the present experiments, the administration of NH_4Cl failed to increase the activity of glutaminases I and II, glutamic dehydrogenase and glutamic oxaloacetic transaminase in dog kidney homogenates. Also the rate of glutamine hydrolysis by intact kidney slices was unchanged by the administration of NH_4Cl. These findings leave unexplained the mechanism for both the increased production of ammonia during acidosis and the increased extraction of glutamine from the renal arterial blood of acidotic dogs as reported by Van Slyke and associates (9).

Although the present studies would indicate that renal glutaminase is not an adaptive-type enzyme system in dogs, Iacobellis, Muntwyler and Griffin (34) have reported that glutaminase activity was increased in the kidneys of two potassium deficient dogs. This is similar to the adaptive increase in enzyme activity noted in hypokalemic rats by Seldin, Rector, Carter and Copenhaver (35) and Iacobellis and co-workers (36). However, the finding of elevated glutaminase activity in potassium deficient dogs could not be confirmed when the activity of glutaminase I in kidney biopsy samples from four hypokalemic dogs was compared with activity in the kidney biopsies from the same dogs during control periods (37).

In summary, the present studies reveal that the adaptation of renal enzymes, which constitutes an important regulatory mechanism of ammonia in rats and guinea pigs, is not a process common to all species. This finding may be of considerable significance with respect to the regulation of ammonia excretion in man. Ammonia excretion in man more closely resembles that of the dog in that the logarithm of ammonia excretion is linearly related to urine pH (8), and urinary ammonia is persistently reduced during chronic carbonic anhydrase inhibition (16). However, more direct studies are needed to clarify the role of the ammonia-producing enzymes in the regulation of ammonia excretion in man.

SUMMARY

An adaptive increase in ammonia excretion following the chronic administration of NH_4Cl is known to occur in the dog. This, as well as the decrease in ammonia excretion following chronic bicarbonate administration, has been confirmed.

The adaptive changes in ammonia excretion are not accompanied by alterations in the activities of any of the renal enzymes known to be involved directly or indirectly in the conversion of glutamine to ammonia. In contrast to findings in the rat, NH_4Cl did not increase the activity of glutaminase I, glutaminase II, glutamic dehydrogenase and glutamic oxaloacetic transaminase in kidney homogenates. Furthermore enzymatic hydrolysis of glutamine by intact kidney slices was unaltered.

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THE ROLE OF pH GRADIENT IN THE DISTRIBUTION OF AMMONIA BETWEEN BLOOD AND CEREBRO-SPINAL FLUID, BRAIN AND MUSCLE *

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The role of ammonia toxicity in the production of the hepatic coma syndrome has not been clearly defined. No direct relationship has been demonstrated consistently between arterial or venous blood ammonia-nitrogen concentration or the arteriovenous ammonia-nitrogen difference and the state of cerebral dysfunction in hepatic disease. Recently a direct relationship between an increase in blood pH and the diffusion of ammonia from blood into tissues has been suggested (2). Other investigators have demonstrated greater toxicity of the more alkaline-ammonium salts (3) and that an increase in blood pH enhanced passage of ammonia into the brain of mice (4).

The diffusion of a weak electrolyte into a cell is determined by the pKa of the weak electrolyte, the pH within and without the cell, and the relative permeability of the cell membrane to the unionized species of the electrolyte (5, 6). It is proposed that ammonia, a weak base with pKa 9.3, should be distributed between blood and cerebrospinal fluid (CSF), brain and muscle according to existing pH gradients, in a manner analogous to that demonstrated in the studies of renal excretion (7, 8), gastrointestinal secretion (9) and absorption (10) of weak acids and bases, and in the studies of distribution of weak organic acids and bases from blood to CSF (11). This study was designed to evaluate in the dog the distribution of ammonia from blood to CSF, brain and muscle under conditions of experimentally induced pH gradients between blood and CSF, brain and muscle.

* A preliminary report of these findings has been published (1).

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METHODS

The principles and methods of a study of the distribution of a drug between blood and cerebrospinal fluid and tissue have been described in detail (12). Fifty-one mongrel dogs lightly anesthetized with pentobarbital were nephrectomized bilaterally. Ammonium bicarbonate solution (0.16 N) was infused intravenously in 48 dogs; ammonium chloride (0.16 N) was infused in 3 dogs. A constant infusion of the ammonium salts was maintained and simultaneous samples of CSF, muscle and blood were taken serially in each animal experiment. Arterial blood samples were obtained through a polyethylene catheter placed in the femoral artery, while venous blood was obtained from jugular bulb and femoral vein catheters. Cerebrospinal fluid (CSF) samples were withdrawn from the cisterna magna through an indwelling percutaneously inserted polyethylene catheter according to the method of Rall, Stabenau and Zubrod (12). The ammonia content of arterial and venous blood and CSF was determined according to the method of Nathan and Rodkey (13) and is expressed as μg. NH_3-N per ml. Muscle biopsy specimens were obtained from the quadriceps and deltoid areas. Brain biopsy samples were aspirated with a 5 mm. bore needle through a parietal craniotomy site before termination of the experiment. Both muscle and brain biopsy samples were immediately frozen in liquid nitrogen and analyzed for their ammonia content according to the method of Nathan and Warren (14). Tissue ammonia content is expressed as μg. NH_3-N per Gm. wet tissue weight.

Glutamine concentrations of whole blood and tissue were determined according to the method of Krebs (15). Blood urea nitrogen concentrations in serum were determined according to the method of Caraway and Fanger (16).

Arterial blood and CSF pH were determined anaerobically on a Beckman model GS pH meter with a Beckman 39022 glass electrode in a 37° C. constant temperature room less than 10 minutes after withdrawal in heparinized syringes. Duplicate determinations bracketed by standards agreed within $\pm$ 0.02 pH unit.

Changes in the pH gradients between blood and CSF, during constant ammonia infusion, were induced and maintained by: intravenous infusion of 0.2 N HCl; intravenous infusion of 0.2 N NaOH; pCO_2 elevation and depression by means of controlled inhalation either of a

TABLE I

The distribution of ammonia from blood to CSF during metabolic and respiratory acidosis and alkalosis *

Condition	NH_4HCO_3 infusion†				
	Control	HCl	NaOH	Respiratory acidosis	Respiratory alkalosis
pH blood‡	7.44 ± 0.008	7.25 ± 0.020	7.60 ± 0.021	7.10 ± 0.022	7.66 ± 0.028
pH CSF‡	7.39 ± 0.012	7.40 ± 0.016	7.41 ± 0.022	7.00 ± 0.022	7.58 ± 0.048
pH gradient	+0.05	−0.15	+0.19	+0.10	+0.08
C_{CSF}/C_{BL}					
obs.§	0.49	0.38 $p<0.01$	0.83 $p<0.01$	0.45 $p=0.5$	0.52 $p=0.6$
calc.‖	1.1	0.7	1.5	1.2	1.2

* C_{CSF}/C_{BL} refers to steady state distribution ratio of ammonia, CSF to blood. The C_{CSF}/C_{BL} observed (obs.) is given as well as the ratio C_{CSF}/C_{BL} calculated (calc.) from observed pH of blood and CSF.

† A constant infusion of 0.16N NH_4HCO_3 was maintained during: control period; infusion of 0.2N HCl (HCl); infusion of 0.2N NaOH (NaOH); inhalation of 20 per cent CO_2 and 80 per cent O_2 mixture (respiratory acidosis); and inhalation of 100 per cent O_2 and CO_2 absorption (respiratory alkalosis). This footnote applies to Tables II, III, IV and VI.

‡ Mean value plus or minus standard error of the mean.

§ The significance of the difference between mean values when compared with the control is given as p.

‖ See Discussion for formula and method of calculation.

mixture of 20 per cent CO_2 and 80 per cent O_2 or of 100 per cent O_2 with CO_2 reabsorption. A closed circuit Rand-Wolfe respirator was utilized.

In addition to separate control animals each dog served as his own control. During the first 90 minutes of infusion a steady state was readily attained in which blood, CSF and muscle ammonia concentration increased, reached a plateau, and then remained essentially the same with the passage of time and constant ammonia infusion. After this period the experimental induction of pH change was begun and maintained for an additional 120 minutes or longer.

Ammonium ion refers to the ionized form of ammonia and unionized ammonia is designated as such, while the term ammonia refers to the total of both ammonium ion, and unionized ammonia. The term C_{CSF} refers to concentration of ammonia in CSF; C_M refers to concentration of ammonia in muscle; C_{BR} refers to concentration of ammonia in brain; and C_{BL} refers to concentration of ammonia in whole arterial blood. The ratio of C_{CSF}/C_{BL},

TABLE II

Distribution of ammonia from blood to muscle and brain during metabolic and respiratory acidosis and alkalosis *

Condition	NH_4HCO_3 infusion				
	Control	HCl	NaOH	Respiratory acidosis	Respiratory alkalosis
Muscle					
pH blood†	7.44 ± 0.008	7.25 ± 0.020	7.60 ± 0.021	7.10 ± 0.022	7.66 ± 0.028
$\frac{A-PV \times 100}{A}$	27.0	7.7 $p<0.01$	41.3 $p=0.05$	25.1 $p=0.8$	34.0 $p=0.5$
C_M/C_{BL}					
obs.‡	0.90	0.73 $p=0.4$	1.8 $p<0.01$	0.73 $p=0.5$	1.5 $p=0.02$
calc.§	2.7	1.8	3.9	1.2	4.4
Brain					
$\frac{A-JV \times 100}{A}$‡	27.1	11.7 $p=0.05$	25.4 $p=0.8$	6.1 $p<0.01$	23.8 $p=0.8$
C_{BR}/C_{BL}					
obs.‡	0.83	0.67 $p=0.2$	2.2 $p<0.01$	1.0 $p=0.2$	2.2 $p<0.01$
calc.§	2.7	1.8	3.9	1.2	4.4

* C_M/C_{BL}, C_{BR}/C_{BL} refer to steady-state distribution ratios of ammonia between muscle and brain and blood. The observed ratios (obs.) are given as well as the ratios C_M/C_{BL} and C_{BR}/C_{BL} calculated from the observed pH of blood and the pH of muscle and brain as 7.0. $\frac{A-PV}{A} \times 100$ and $\frac{A-JV}{A} \times 100$ represent A–V ammonia concentration differences between arterial (A) and peripheral (PV) and jugular venous (JV) blood, respectively.

† Mean value plus or minus standard error.

‡ The significance of the difference between mean values when compared with the control is given as p

§ See Discussion for formula and method of calculation.

C_M/C_{BL}, or C_{BR}/C_{BL} refers to the distribution ratio of ammonia at steady state or near steady state.

The arterial (A) whole blood ammonia concentration minus venous (V) whole blood ammonia concentration (A-V difference) was expressed as the ratio of the difference A-V to A, times 100

$$\left(\frac{A-V}{A} \times 100\right).$$

Statistical evaluation of the data is expressed as the standard error of the mean (S.E.). The symbol ± refers to the S.E. The "t" ratio was used to obtain the p values (17).

RESULTS

1. *Ammonia distribution during acidosis and alkalosis*

Steady state ratios relating concentration of ammonia in CSF (C_{CSF}/C_{BL}), muscle (C_M/C_{BL}) and brain (C_{BR}/C_{BL}) to concentration of ammonia in whole arterial blood were determined during various phases of the study and were compared to those ratios achieved during a control infusion state (Tables I and II).

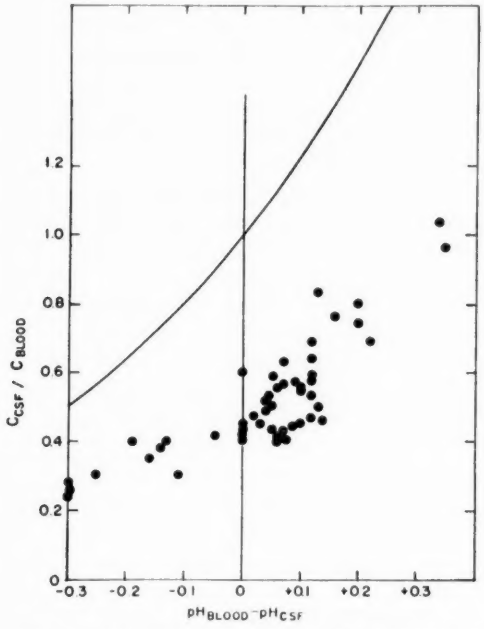

FIG. 1. THE RELATION BETWEEN pH GRADIENT AND THE OBSERVED AND CALCULATED DISTRIBUTION RATIO OF AMMONIA, CSF TO BLOOD (C_{CSF}/C_{BL})

Observed C_{CSF}/C_{BL} ratios at steady state are plotted as solid dots against the pH gradient derived from the blood and CSF pH observed during the steady state. The solid line curve represents the locus of plotted points for the predicted C_{CSF}/C_{BL} as calculated from the observed blood and CSF pH.

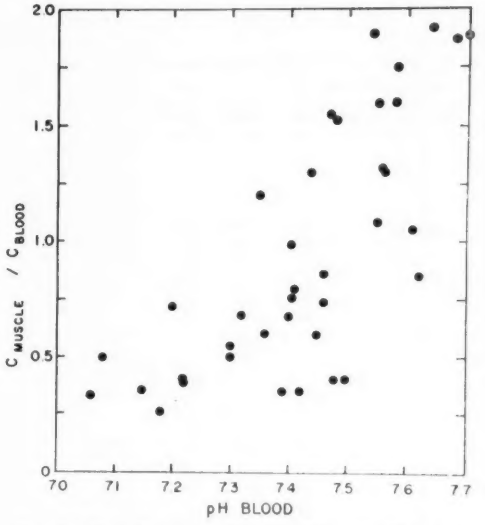

FIG. 2. THE RELATION BETWEEN BLOOD pH AND THE OBSERVED DISTRIBUTION RATIO OF AMMONIA, MUSCLE TO BLOOD (C_M/C_{BL})

Observed C_M/C_{BL} ratios at steady state are plotted as solid dots against the pH of blood observed during the steady state.

A. Cerebrospinal fluid. C_{CSF}/C_{BL} decreased significantly from the control infusion value of 0.49 to 0.38 during metabolic acidosis while during metabolic alkalosis there was a significant increase to 0.83. Respiratory acidosis and alkalosis caused no significant variation from control infusion ratio.

The mean values for blood and CSF pH are shown in Table I. Change in magnitude and or direction of pH gradient occurred only during the infusion of NaOH or HCl due to changes in blood pH without significant alteration of CSF pH. During respiratory pCO_2 changes, a pH gradient similar to that in the control state was maintained due to simultaneous changes of the blood and CSF pH. There was a direct relation between the magnitude and the direction of the pH gradient produced and the distribution of ammonia to the CSF. This is shown in Figure 1. When a large positive pH gradient (pH blood

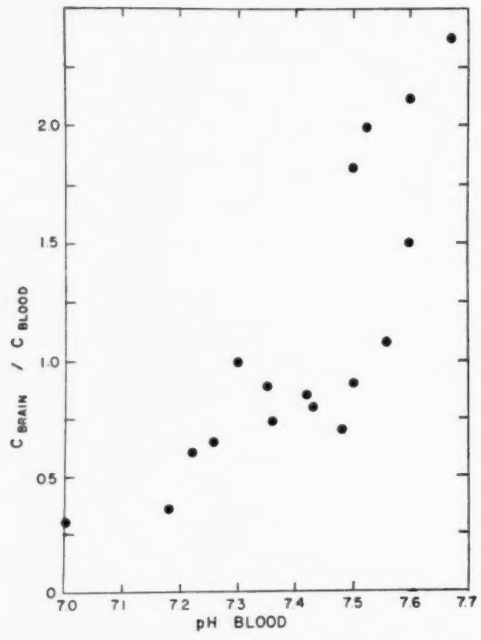

FIG. 3. THE RELATION BETWEEN BLOOD pH AND THE OBSERVED DISTRIBUTION RATIO OF AMMONIA, BRAIN TO BLOOD (C_{BR}/C_{BL})

Observed C_{BR}/C_{BL} ratios at steady state are plotted as solid dots against the pH of blood observed during the steady state.

minus pH CSF) existed an increase in C_{CSF}/C_{BL} occurred, and when a large negative gradient existed a decrease in the C_{CSF}/C_{BL} occurred.

During metabolic acidosis and alkalosis samples of aqueous humour and CSF were withdrawn simultaneously. The ratio of the concentration of ammonia in aqueous humour to the concentration of ammonia in blood in six dogs so examined was quantitatively similar to the ratio C_{CSF}/C_{BL}.

B. Tissues. The decrease of C_M/C_{BL} from control value of 0.90 during metabolic and respiratory acidosis was not significant in a comparison of group means (Table II), but was significant in a paired comparison within each experiment ($p = <0.05$). A significant increase to 1.8 during metabolic alkalosis and to 1.5 during respiratory alkalosis occurred. The direct relationship between blood pH and C_M/C_{BL} is demonstrated in Figure 2.

The data in Table II demonstrate the relation between the observed A-PV difference (arterial whole blood ammonia concentration minus peripheral venous whole blood ammonia concentration) and A-JV difference (arterial whole blood ammonia concentration minus jugular venous whole blood ammonia concentration) to the relative distribution of ammonia between blood and muscle and brain. During NaOH infusion muscle ammonia concentration increased and A-PV difference was significantly greater than during the control period. With HCl infusion the muscle ammonia concentration decreased and A-PV difference significantly decreased. Respiratory acidosis and alkalosis produced no significant variation in A-PV difference.

C_{BR}/C_{BL} increased significantly during metabolic alkalosis (2.2) and respiratory alkalosis (2.2) from the control infusion ratio of 0.83. There was an insignificant decrease in C_{BR}/C_{BL} to 0.67 during metabolic acidosis and increase to 1.0 during respiratory acidosis. Animals exposed to respiratory acidosis after periods of respiratory alkalosis had a change of C_{BR}/C_{BL} from 2.0 in alkalosis to 1.3 ($p = 0.02$) in acidosis. The direct relationship between blood pH and C_{BR}/C_{BL} is demonstrated in Figure 3.

Significant narrowing of the A-JV difference

TABLE III

Brain and muscle ammonia concentration as related to conditions of pH change as well as time and amoun. of ammonia infused

Condition	NH_4HCO_3 infusion				
	Control	HCl	NaOH	Respiratory acidosis	Respiratory alkalosis
Brain NH_3–N, μg./Gm.*	11.3 ± 2.5	8.1 ± 1.1	49.4 ± 7.2	31.3 ± 2.2	60.0 ± 2.0
Muscle, μg. NH_3–N/Gm.*	12.3 ± 3.5	8.5 ± 1.8	28.9 ± 1.2	28.3 ± 9.6	47.0 ± 8.0
NH_3–N infused, μg./Kg./min.*	609.8 ± 23.4	570.2 ± 68.3	541.4 ± 57.1	585.3 ± 66.5	567.5 ± 47.4
Duration NH_4HCO_3 infusion* (min.)	313.8 ± 20.4	263.2 ± 39.9	262 ± 31.6	365 ± 31.8	300 ± 30.0

* Mean value plus or minus standard error of the mean.

TABLE IV

Representative typical experiments during steady state ammonia infusion with variation of blood pH by metabolic and respiratory means

Condition*	Time	Ammonia concentration						pH		Distribution ratios		
		Art.†	J.V.‡	P.V.§	CSF	Mus.‖	Br.¶	Blood	CSF	C_{CSF}/C_{BL}	C_M/C_{BL}	C_B/C_{BL}
	min.	*μg./100 ml.*				*μg./100 Gm.*						
Expt. 1 (10.1 Kg.)** constant infusion 6.5 mg. NH_3–N/min.												
Control	60	700			315	250		7.50	7.44	0.45	0.36	
	90	730		610	321	490		7.50	7.45	0.44	0.67	
HCl, 0.48 mEq./min.	60	1,260	980	1,130	406	290		7.28	7.48	0.32	0.23	
	120	1,420	1,070	880	436	150		7.12	7.49	0.31	0.11	
	180	1,770	1,290	1,220		600	820	7.11	7.46		0.34	0.46
Expt. 2 (11.4 Kg.) constant infusion 7.4 mg. NH_3–N/min.												
Control	60				284	290						
	120	753	428	452	297	730		7.49	7.43	0.40	0.97	
NaOH, 0.51 mEq./min.	60	940			490	2,100		7.75	7.52	0.52	2.2	
	180	1,740	1,480	1,370	1,190	3,260	3,600	7.70	7.48	0.69	1.9	2.1
Expt. 3 (10.2 Kg.) constant infusion 7.6 mg. NH_3–N/min.												
Control	90	855	578	780	444	556		7.49	7.42	0.52	0.65	
	120	1,015	650	630	436	757		7.47	7.42	0.43	0.75	
NaOH, 0.35 mEq./min.	45	1,895	1,200	885	963	1,290		7.58	7.50	0.51	0.68	
	105	1,510	1,150	1,180	1,060	2,710		7.60	7.50	0.70	1.80	
HCl, 0.70 mEq./min.	45	2,590			919	2,310	4,000	7.30	7.29	0.36	0.93	1.5
Expt. 4 (11.5 Kg.) constant infusion 3.8 mg. NH_3–N/min.												
Control	60	416	248	228	133	410		7.43	7.35	0.32	0.99	
	90	420		213	167	394		7.43	7.36	0.40	0.94	
Inhalation 100% O_2;	60	326	246	162	198	204		7.63	7.49	0.61	0.63	
CO_2 absorption	90	313	249	224	231	334		7.63	7.43	0.74	1.06	
Inhalation 20% CO_2	60	485	460	368	234	207		7.23	7.07	0.48	0.43	
+ 80% O_2	120	403	430	402	255	328	567	7.09	6.99	0.63	0.81	1.4

* Continuous infusion of 0.16N NH_4HCO_3 was maintained during all phases of each experiment.
† Arterial blood.
‡ Jugular vein blood.
§ Peripheral vein blood.
‖ Muscle.
¶ Brain.
** The weight of each dog is given after the experiment number.

during both metabolic and respiratory acidosis occurred. Metabolic or respiratory alkalosis, however, produced no significant variation from control A-JV differences though relative ammonia concentrations in brain were increased. On the whole, A-PV differences were greater than A-JV differences during the experimental phases. Three dogs infused with NaOH had negative A-JV differences during a convulsive state. The only other occurrences of negative A-V differences were during HCl infusion.

The magnitude of tissue ammonia concentration was not related to the amount and duration of ammonia infusion (Table III). A comparison was made of mean brain and muscle concentration in several animals selected from the various categories because of similarity of weight and duration of experiment. The amounts of ammonia infused as ammonium bicarbonate in each state was similar. The average amount of NH_3–N infused was 575 ± 52.0 μg. per Kg. per minute for periods of about five hours. Though the amount of ammonia infused in each state was quite similar, brain and muscle concentrations varied markedly in the various experimental states. A high incidence of mortality occurred in dogs with a mean brain level above 40 μg. NH_3–N per Gm.

Details of the effects of pH gradient changes on

ammonia distribution in individual experiments can be obtained from Table IV. Each animal experiment is recorded in its entirety. Each dog acted as his own control. Mean ammonia-nitrogen concentrations obtained from other animals before NH_4HCO_3 infusion were: 91.1 ± 13.2 μg. per 100 ml. for blood; 35.5 ± 3.7 μg. per 100 ml. for CSF; 293.0 ± 31.2 μg. per 100 Gm. for muscle; and 353.0 ± 52.2 μg. per 100 Gm. for brain ($N = 8$). Marked increase in CSF, muscle and brain ammonia concentration occurred during NaOH infusion (Experiment 2) where large positive pH gradients were produced. In contrast there was a decrease in tissue and CSF ammonia concentration during HCl infusion (Experiment 1) where large negative pH gradients were produced. Similar but less marked changes are noted in a dog which received acid infusion after NaOH infusion (Experiment 3). Experiment 4 demonstrated changes in ammonia distribution which occurred during respiratory alkalosis followed by respiratory acidosis.

II. The pH gradient variation produced by acidosis and alkalosis

During the preinfusion state blood pH was 7.43 ± 0.011 and the CSF pH was 7.35 ± 0.012. During the control infusion the pH of blood was 7.44 ± 0.008, and CSF pH was 7.39 ± 0.012. The mean pH values of blood and CSF during the various states of pH change (Table I) reveal that during acid and alkali infusion CSF pH varied only slightly while blood pH decreased and increased. Blood pH and CSF pH decreased and increased simultaneously when acidosis and alkalosis were produced by varying pCO_2. When the respiratory pH changes were induced during NH_4HCO_3 infusion blood pH remained alkaline to CSF pH.

Table V presents experiments in four nephrectomized dogs and one dog with intact kidneys. Two or more similar experiments were performed in each case. These experiments demonstrate the changes in CSF and blood pH during respiratory alkalosis and acidosis in an animal receiving no infusion, animals receiving a solution such as antipyrine which does not apparently affect pH, and in animals receiving NH_4HCO_3 infusion. It appears that pH differences between blood and CSF may vary markedly in magnitude and direction depending upon the nature of a combined metabolic and respiratory acidosis or alkalosis.

Steady states in which arterial blood concentrations remain at a constant level during acidosis or alkalosis were difficult to maintain. We have observed, during constant NH_4HCO_3 infusion in the nephrectomized dogs as well as in dogs with intact kidneys, there was an immediate large and sustained rise in arterial ammonia concentration from control levels upon administration of intravenous

TABLE V

The effect of pCO_2 changes on the pH of blood and CSF during states of mild metabolic acidosis and alkalosis

Experiment no.	I.V. solution	pH	No infusion	Infusion: Control* 60	Control* 120	Respiratory alkalosis* 60	Respiratory alkalosis* 90	Respiratory alkalosis* 120	Respiratory acidosis* 60	Respiratory acidosis* 90	Respiratory acidosis* 120
5	None†	Blood	7.43	7.45		7.49	7.51		6.93	6.94	
		CSF	7.35	7.36		7.49	7.51		7.03	7.00	
6	Antipyrine	Blood	7.35		7.38				7.02		6.93
		CSF	7.23		7.30				7.01		7.04
7	Antipyrine	Blood	7.39		7.40	7.61	7.73	7.66			
		CSF	7.29		7.32	7.61	7.66	7.63			
8	NH_4HCO_3	Blood	7.34	7.43	7.44	7.63	7.63	7.59	7.23	7.21	7.09
		CSF	7.25	7.25	7.26	7.49	7.43	7.46	7.07	7.06	6.99
9	NH_4Cl	Blood	7.42	7.30	7.29	7.60		7.58	6.96		6.93
		CSF	7.33	7.33	7.35	7.70		7.72	7.08		7.09

* Duration of experimental period is given in minutes.
† All dogs were nephrectomized with the exception of Dog 5.

TABLE VI

Glutamine concentration in blood, muscle and brain

Condition	No infusion*	NH_4HCO_3 infusion				
		Control†	HCl†	NaOH†	Respiratory acidosis†	Respiratory alkalosis†
Arterial blood concentration						
NH_3–N, μg./100 ml.	91.0 ±3.2	87.0	675	930	3,720	1,550
Glutamine, mg./100 ml.	11.6 ±0.5	7.0	8.7	6.1	5.4	1.8
Muscle concentration						
NH_3–N, μg./100 Gm.	293.0 ±31.2	950	760	1,050	3,540	2,870
Glutamine, mg./100 Gm.	57.8 ±8.7	89.5	130	95.8	86.0	120.0
Brain concentration						
NH_3–N, μg./100 Gm.	353 ±52.3	900	420	820	3,850	1,020
Glutamine, mg./100 Gm.	52 ±4.6	141	83.2	85.8	74	114

* Mean values plus or minus standard error of the mean.
† Values from each experiment.

HCl and NaOH and during inhalation of 20 per cent CO_2 and 80 per cent O_2 mixture, but not during inhalation of 100 per cent O_2 with CO_2 absorption. The rise of blood ammonia during acidosis appeared to be secondary to accumulation of ammonia due to the reduced passage into tissues. The rise following the infusion of NaOH suggested the possibility that pH change decreased enzymatic removal of ammonia either by decreasing formation of urea in the liver or of glutamine in muscle and brain. Preliminary study of blood urea nitrogen (BUN) elevation in seven nephrectomized dogs demonstrated no striking difference in the rate of BUN elevation between control infusion state and states of experimental pH change, nor was there any relation between the BUN and the blood ammonia-nitrogen concentration. A preliminary study of blood and tissue glutamine concentration involving six nephrectomized dogs has revealed blood glutamine concentrations which were all consistently lower during ammonia infusion than before infusion was begun (Table VI). While a fairly constant rise in muscle glutamine (11.3 ± 1.3 mg. per 100 Gm. per hour) occurred during control ammonia infusion, an irregular change in muscle glutamine content occurred during induced pH changes. Although there appears to be marked glutamine production during ammonia infusion there is no direct relation between the glutamine concentration and the ammonia concentration in blood, muscle or brain.

DISCUSSION

The relation between ammonia toxicity, hepatic disease, and cerebral dysfunction has been recently reviewed by Warren (3). The index of ammonia toxicity in hepatic disease has always been in terms of blood concentration with the assumption that tissue concentration was a direct function of blood ammonia concentration (18–24).

Gyorgyi and Kleinschmidt (25) in 1926 noted that Eck fistula dogs in meat intoxication were usually alkalotic and that they improved when given hydrochloric acid *per os*. Vanamee and co-workers (26) and Robin, Whaley, Crump and Travis (27) have recently shown that the majority of patients in hepatic coma are in a respiratory alkalosis and have suggested that alkalosis might enhance ammonia toxicity. Lawrence and co-workers (2) have hypothesized an increased passage of ammonia into the tissue with an increase in blood pH, and have attempted to prove this by studying A-V differences. War-

ren and Nathan (4) demonstrated an increased rate of passage of ammonia into the brain of the mouse with a rise in blood pH.

Some clinical confirmation of the experimental findings have been published. Recent studies on blood A-V ammonia differences in patients with elevated blood ammonia concentrations appear to contradict the assumption that tissue uptake is always proportional to blood ammonia concentrations (28, 29). Occasional case reports have appeared in the literature of patients given intravenous NH_4Cl for severe alkalosis with the consequent development of symptoms of ammonia toxicity (30, 31).

The purpose of this study was to investigate the role of pH gradients between blood and CSF, brain and muscle in the distribution of ammonia from blood to the cerebrospinal fluid and those tissues. Milne, Scribner and Crawford (32) have reviewed the use of a pH gradient drug distribution hypothesis in renal tubular excretion and absorption, gastrointestinal secretion and absorption and pancreatic secretion of weak organic acids and bases. Waddell and Butler (8) demonstrated the role of pH in controlling the passage of an organic acid, phenobarbital, into the brain. Stabenau, Rall and Zubrod (11) demonstrated that certain acidic and basic compounds with pKa near the physiologic pH range had distribution ratios between plasma and CSF which are related to a normal pH gradient between blood and CSF, and that experimental changes in that gradient caused predictable changes in such distribution. In addition these authors discussed in detail the application of the pH gradient hypothesis to the transfer of drugs and metabolites into the cerebrospinal fluid.

The pH gradient drug distribution hypothesis explaining the transfer of weak electrolytes from blood to CSF, brain and other tissues is essentially that amounts of ionizable compounds existing in two fluid compartments separated by a membrane completely permeable to the unionized species of the compound and relatively impermeable to the ionized species will be distributed unequally when a pH gradient exists between the compartments. In the case of ammonia, at steady state, the greater concentration of total compound should be on the side of the membrane where the pH is lower. The ratio of total ammonia between CSF and blood should be the inverse ratio of the per cent unionized in each phase (11, 32).

The data in Table I have demonstrated that the pH gradient between blood and CSF varies from that of the control infusion state during alkalosis and acidosis. The direct effect of the direction as well as magnitude of such gradients on the steady state distribution ratios of ammonia between blood and CSF, brain and muscle can be calculated by using an equation first utilized by Jacobs (6) and later by others (32) to equate distribution ratios of ionizable compound to the unionized state:

$$C_{CSF}/C_{BL} = \frac{1 + 10^{pKa-pH\ CSF}}{1 + 10^{pKa-pH\ BL}}.$$

By comparing the ratio of unionized ammonia in blood to that in CSF, as calculated from their respective pH, to the observed ratio C_{CSF}/C_{BL}, the role of pH gradient in ammonia distribution becomes apparent. In Figure 1 it is demonstrated that the calculated C_{CSF}/C_{BL} for the pH values in the experiments is about twice that of the observed ratios through the range of pH gradient -0.3 to $+0.3$ pH unit. Milne and associates (32) have considered in detail several of the reasons why observed ratios in such a study as this would necessarily be lower than predicted ratios. Some unknown degree of permeability to the ionized molecular species may exist, leading to a falsely high calculated ratio; a rate limitation of the diffusion process itself may occur; and apparent reduction of diffusion concentration ratios may occur due to reference to arterial instead of capillary plasma concentration.[1]

Simultaneous blood and CSF pH determinations as presented in this communication and in other studies (11) confirm observations of Cestan, Sendrail and Lasalle (33) and Leusen (34) that during metabolic acidosis and alkalosis the blood pH decreased and increased, respectively, while CSF pH remained unchanged. During respiratory acidosis and alkalosis blood and CSF pH decreased and increased simultaneously. A demonstration of combined metabolic and respiratory acidosis and alkalosis with resultant CSF and

[1] It should be noted that due to technical difficulties arterial whole blood ammonia-nitrogen concentration was used in this study.

blood pH changes is given in Table V. The presence of the excess of bicarbonate ion in the ammonium bicarbonate infusion lead to a blood pH more alkaline than CSF pH during both respiratory acidosis and alkalosis. When NH_4HCO_3 was infused under similar conditions the blood pH became less alkaline than CSF pH during both respiratory acidosis and alkalosis. From these data one may expect to have a small but significant pH gradient between blood and CSF during induced respiratory alkalosis or acidosis only when a previous metabolic acidosis or alkalosis is present. These findings suggest that there is delayed diffusion of infused chloride or bicarbonate ion between blood and CSF but a rapid equilibration of CO_2. This presents a situation quite similar to that described by Wallace, Hastings and Lowry (35, 36) for the muscle cell under similar experimental consideration.

Muscle intracellular pH according to Wallace and Hastings is 6.93 ± 0.12. Infusion of HCl produced serum pH decrease while calculated muscle pH remained the same. With $NaHCO_3$ infusion serum pH was raised and muscle pH remained the same or decreased slightly (35). Brodie and Woodbury (37) estimated normal rat cerebral cortical intracellular pH to be 7.04 ± 0.07 while plasma pH was 7.41 ± 0.03. When 30 per cent CO_2 was inhaled, plasma pH decreased to 6.95 ± 0.01 and cerebral pH decreased to 6.75 ± 0.06. In Table II the calculated distribution ratios for ammonia between blood and brain are seen to correlate in extent and magnitude of variation to the observed distribution ratios when the pH of muscle and brain is assumed to be 7.0, though with pCO_2 elevation and depression tissue pH value may decrease and increase to some extent. From our data utilizing a pH gradient distribution hypothesis, the steady state distribution of ammonia from blood to brain and muscle appears to be dependent upon a pH difference between blood and the tissues. This is substantiated by the above observations concerning the role of CSF pH (which can be measured) in the distribution of ammonia to CSF. CSF pH appears to undergo little change in metabolic acidosis and alkalosis, but does change when pCO_2 of blood is experimentally varied. Muscle and brain pH changes may well be similar to those in CSF. It has been suggested recently that CSF is not similar to extracellular fluid but has characteristics of intracellular fluid (11).

Although ammonia is metabolized to glutamine and other compounds in muscle and brain, measurements of tissue ammonia performed during a steady state situation appear to be meaningful. Tissue ammonia concentrations reflect changes in ammonia passage into cells by diffusion in a manner analogous to passage into CSF where no metabolism is thought to occur.

This study emphasizes that knowledge of arterial blood pH is necessary in order to evaluate fully the relationship between arterial ammonia concentration and ammonia toxicity in hepatic coma. Though ammonia elevation in the central nervous system and muscle would appear to explain some of the features of hepatic coma, other compounds, *e.g.*, amino acids of relative availability, due to defects in liver anabolism or detoxication, may be abnormally distributed between blood and central nervous system and muscle due to gradients in pH. Acidic compounds with certain ionization characteristics may be less able to pass into tissues during states of alkalosis. Thus if any corrective therapy utilizing metabolic and respiratory acidosis is found useful, it may be due to alteration in the transfer of such compounds to muscle and central nervous system.

SUMMARY

1. In an effort to delineate the role of pH in the distribution of ammonia between blood and various body fluids and tissues, temporary pH gradients between blood and cerebrospinal fluid, brain and muscle were experimentally induced by means of intravenous infusion of hydrochloric acid and sodium hydroxide solution or elevation and depression of the partial pressure of carbon dioxide through respiratory means. Simultaneous brain, muscle and cerebrospinal fluid ammonia concentrations were serially determined during steady state conditions and were related to arterial whole blood ammonia concentrations at corresponding times.

2. A direct relation was observed between the diffusion of ammonia into cerebrospinal fluid and the magnitude and direction of a gradient in pH between blood and cerebrospinal fluid.

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SUMMARY

1. In an effort to delineate the role of pH in the distribution of ammonia between blood and various body fluids and tissues, temporary pH gradients between blood and cerebrospinal fluid, brain and muscle were experimentally induced by means of intravenous infusion of hydrochloric acid and sodium hydroxide solution or elevation and depression of the partial pressure of carbon dioxide through respiratory means. Simultaneous brain, muscle and cerebrospinal fluid ammonia concentrations were serially determined during steady state conditions and were related to arterial whole blood ammonia concentrations at corresponding times.

2. A direct relation was observed between the diffusion of ammonia into cerebrospinal fluid and the magnitude and direction of a gradient in pH between blood and cerebrospinal fluid.

3. There appeared to be a direct and predictable correlation between alteration of blood pH and the tissue ammonia concentration. During metabolic and respiratory alkalosis brain and muscle ammonia concentrations increased two- to threefold, while during metabolic and respiratory acidosis brain and muscle concentrations remained at or decreased below control concentrations.

4. Explanation for these findings may be found in the pH gradient drug distribution hypothesis.

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TRANSCORTIN: A CORTICOSTEROID-BINDING PROTEIN OF PLASMA *†

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The general field of the binding of steroids and their conjugates to human plasma proteins has recently been reviewed by the authors (1). Evidence was presented that Fractions IV-1, IV-4 and V (obtained by the Cohn fractionation procedure) were mainly responsible for the binding, and hence transport, of the unconjugated steroids. The binding of conjugates of steroids to the plasma protein was also shown to be considerable. Furthermore, evidence was obtained that cortisol and corticosterone were avidly bound by a "special" protein in human plasma. This conclusion was based on the fact that cortisol and corticosterone were bound to unfractionated human plasma much more strongly than determinations of binding to alcohol-fractionated human plasma proteins had led us to expect. In fact, these two corticosteroids were bound at least as strongly as estrone and estradiol to plasma diluted 1:4 or 1:40 [see Table VII of (1)]. The existence of such a "special" protein has been independently described by Daughaday (2–5). At first he attributed all the binding to albumin; that observed in Fraction IV-4 (α-globulin) was assigned to its content of albumin (6, 7). Later (2), using the same techniques but employing a radioactive label for assay instead of a chemical determination, he concluded that there was in the α-globulin fraction of human plasma from cortisone-treated patients (but not normal subjects) a protein low in capacity but high in affinity for corticosteroids. This is in contrast to albumin which has a high capacity but low affinity for corticosteroids (1). In subsequent papers (3–5) he concluded that normal subjects also possess corticosteroid-binding protein.

Some time ago Roberts and Szego (8) concluded that Fraction III-0 was important in the binding and transport of estrogens in human blood, a finding which was challenged by Bischoff, Stauffer and Gray (9). In our studies we were unable to demonstrate the binding of estrogens to Fractions III-0 and recent work by Bischoff and Stauffer (10) is in agreement with these findings, pointing to albumin as the major protein concerned with the transport of estrogens.

In this paper we wish to present further data pointing to the existence of a special protein (or proteins) with great affinity for the binding of cortisol and corticosterone. By analogy to transferrin, the iron-binding protein of plasma, we have named the corticosteroid-binding protein "transcortin." Additionally, evidence will be presented that the concentration of this protein is considerably increased during the third trimester of pregnancy. Data will also be shown which indicate the lack of such a protein for the binding of estrogens in human plasma, from both normal and pregnant subjects.

METHODS AND MATERIALS

Equilibrium dialysis was performed according to methods previously reported (1). Since previous studies indicated that undiluted plasma bound 99 per cent of the C^{14}-corticosteroid added, dilution of plasma gave results which made differences in the binding among the various steroids more pronounced and easier to quantitate. It was also established that the presence or absence of stabilizers in human serum albumin (HSA) did not affect the binding of the various steroids to HSA or plasma, a finding observed by Bischoff and Stauffer for the estrogens (10). Hence, for the majority of the experiments salt-poor HSA supplied in solution by the American Red Cross was used. Free electrophoresis showed the HSA to be homogenous. It was also shown that when plasma and HSA were diluted with phosphate buffer (pH 7.4, 0.05 M), the binding of cortisol did not differ from that observed when 0.15 M saline was used for dilution. The method, therefore, consisted of placing 2 ml. of plasma di-

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luted to 10 ml. with 0.15 M saline, in 23/32" NoJax Visking casing. The casing was placed in a 125 ml. Erlenmeyer flask containing 29 ml. of either 1 per cent HSA (diluted with saline) or saline and 1 ml. of radioactive steroid dissolved in saline. The flasks were rocked slowly for 72 or more hours at 4° C. to assure equilibration of these steroids. Small amounts of penicillin were added to each flask (10,000 units) following studies which indicated the antibiotic did not interfere with the binding of any of the steroids studied.

Blood (30 to 50 ml.) was collected in 0.5 ml. of heparin (4 mg. per ml.) and the erythrocytes separated from the plasma (use of serum did not alter the results). The blood samples were obtained in the late morning or early afternoon from normal pre-menopausal subjects and pregnant women. The plasma was used the day on which the blood was drawn.

C^{14}-labeled steroids were added in amounts ranging from 0.25 to 0.5 μg. per flask (*ca.* 2,000 cpm). Carrier steroid was added dissolved in small amounts of ethanol, the concentration of the alcohol never exceeding 0.1 per cent. Both the carrier and radioactive steroids were 99 per cent or more pure as judged by infrared spectroscopy and/or paper chromatography. Deoxycorticosterone acetate and cortisone acetate were converted to the free alcohols by incubation with acetyl cholinesterase or by chemical hydrolysis.

Following dialysis the contents of the cellophane bag and of the flask were separately extracted three times with two volumes of chloroform. The chloroform extracts were dried by air, plated on steel planchettes and counted in a windowless gas flow counter. The weight of the samples was negligible and no correction was necessary for self-absorption of radioactivity. The percentage of binding of steroids to protein was determined by the following Equation *1*):

$$1) \qquad \% \text{ bound} = 100\left(1 - \frac{D \cdot V_R}{R \cdot V_D}\right)$$

where R and D are the amounts of radioactivity present inside and outside the dialysis casing, respectively, and V_R and V_D are the corresponding volumes.

The recovery of the added C^{14}-steroids ranged from 85 to 100 per cent. No binding of the steroid occurred to the glassware or to the dialysis casing. In several experiments it was shown that under the conditions of the dialysis the added C^{14}-cortisol was recovered unchanged as judged by paper chromatography. The complete procedure was performed in triplicate and the results represent the average of the three determinations which seldom varied more than 3 percentage points from the average.

Certain samples were submitted to continuous flow paper electrophoresis on the Spinco Model CP apparatus using Schleicher and Schuell No. 470 paper. Two buffer systems were used: barbital buffer, 0.02 M, pH 8.6 and acetate buffer, 0.02 M, pH 5.2. A potential of 660 to 800 V. was applied, producing a current flow of 40 to 60 ma. Usually 25 ml. of serum was equilibrated against the buffer overnight and the small amount of sediment formed centrifuged down before application of the serum to the paper at the rate of *ca.* 1 ml. per hour. The volumes of the 32 fractions varied; each tube was diluted to 15 ml. with buffer. The optical density at 280 mμ was determined using a Beckman Model DU spectrophotometer. Protein analysis was performed using a Spinco Model R paper electrophoresis apparatus. The binding of 4-C^{14}-cortisol was determined on each fraction by means of equilibrium dialysis. For this purpose 5 ml. of each fraction diluted with 5 ml. of saline was used. After dialysis the samples containing barbital buffer were adjusted to pH 9 to 10 to avoid the extraction of diethylbarbituric acid, which, if present, gave significant weight to the samples.

The optical density was converted to concentration using a separate factor, based on Kjeldahl determinations kindly performed by Mr. Kenneth Buchwald of this Institute, for each protein fraction. From this the amount of binding per mg. of protein was calculated.

RESULTS

Earlier work (1) showed that undiluted plasma bound trace amounts of added C^{14}-labeled cortisol and corticosterone essentially quantitatively. Therefore, in order to be able to demonstrate increases in extent of binding, dilution of the plasma was necessary. Dialyses of diluted plasma (1 : 5 with 0.15 M saline) against 0.15 M saline showed, however, only minor differences in the binding of the various classes of steroids. We therefore sought to cancel the effect of the albumin on binding by dialyzing against a concentration of albumin equal to that in diluted plasma. Since the albumin concentration in the plasma is usually approximately 5 Gm. per 100 ml., dialyses of plasma diluted 1 : 5 were performed against 1 Gm. per 100 ml. HSA.

TABLE I

Demonstration of corticosteroid-binding protein other than albumin in human plasma

C^{14}-Steroid	% Bound*
Cortisol	89, 88, 82
Corticosterone	72, 78
11-Deoxycorticosterone	77
Cortisone	62
Pregnane-3,11,20-trione	63
Progesterone	20, 15
Δ^4-Androsten-11β-ol-3,17-dione	18
Testosterone	11, 10
Estrone	0, 0
Estradiol	0, 0
Estriol	12

* Dialysis of plasma from clinically normal individuals diluted 1:5, against 1 per cent HSA. Each number is the average of triplicate determinations done with plasma from three subjects.

TABLE II

Demonstration of a corticosteroid-binding protein other than albumin in human plasma *

Subject	Condition	Dialyzed against	Steroid†	% Bound
Pe.	Pregnant	Saline	F	95
Pe.	Third trimester	1% HSA	F	94
Cr.	Third trimester	Saline	F	94
Cr.	Third trimester	1% HSA	F	93
Cr.	Third trimester	Saline	B	93
Fa.	Third trimester	Saline	F	96
Fa.	Third trimester	1% HSA	F	94
Fa.	Third trimester	Saline	B	94
Ke.	Third trimester	Saline	F	95
Ke.	Third trimester	1% HSA	F	96
Py.	Third trimester	Saline	F	95
Py.	Third trimester	1% HSA	F	94
Wa.	Normal female	Saline	Estriol	77
Wa.	Normal female	1% HSA	Estriol	0

* Dialysis of plasma diluted 1 : 5.
† F, cortisol; B, corticosterone.

The results of a series of such experiments with various steroids are shown in Tables I and II. Binding varies from zero for the estrogens to 80 to 90 per cent for cortisol (Table I). The same results were obtained for estrone and cortisol by dialysis in the opposite direction. As Daughaday (4) observed, slight alterations in the cortisol molecule decrease the ability of the molecule to bind to transcortin. Because the binding of cortisol to transcortin is so much stronger than that to albumin, dialysis against albumin usually lowered the amount of binding by only one or two percentage points as compared to dialysis against physiological saline (Table II). On the other hand, the value for estriol dropped to zero, indicating binding exclusively to albumin.

From Tables I and II it can be seen that corticosterone binds nearly as effectively as cortisol.

TABLE III

Competition experiments with cortisol (F) and corticosterone (B)

C^{14}-Steroid	Carrier F	% Bound*	Carrier B	% Bound*
F		61		
	1 μg.	44	1 μg.	49
	3	22	3	31
	5	15	5	30
B				86
	1	62	1	67
	3	59	3	61
	5	57	5	59

* This is the per cent of the radioactive steroid bound to pooled plasma proteins from normal subjects diluted 1:5 and dialyzed against 1 per cent HSA.

Competition experiments were performed to determine if the sites of binding of the two steroids were identical. The results shown in Table III demonstrate that cortisol is more effective than corticosterone in inhibiting the binding of radioactive cortisol whereas these two steroids are nearly equally effective in inhibiting the binding of radioactive corticosterone.

The binding curve of cortisol to plasma is shown in Figure 1. This was obtained by determining the binding of C^{14}-cortisol with the addition of 0 to 10 μg. of carrier cortisol. From the equilibrium equation for association of cortisol with plasma protein (Equation *2*),

$$2) \qquad K = \frac{(Pr \cdot C)}{(Pr)\ (C)}$$

the following equation can be derived:

$$3) \qquad \frac{1}{(Pr \cdot C)} = \frac{1}{K\ (Pr_t)\ (C)} + \frac{1}{(Pr_t)}$$

where (Pr_t) is the total concentration of protein, (Pr) and (C) are the concentrations of unbound protein and cortisol, respectively, and $(Pr \cdot C)$ is the concentration of protein-bound cortisol. A plot of $1/(Pr \cdot C)$ *versus* $1/(C)$ will be a straight line if the equilibrium constant K is the same for all sites (homogeneity of sites). Deviation of the binding curves from linearity reflects the heterogeneity of the sites for cortisol. From the intercept of the y axis, it was calculated that (Pr_t) was 3.2×10^{-7} M per L. and 1.3×10^{-7} M per L. for the pregnancy and normal plasmas, respec-

tively. These values are actually the concentration of sites. The protein concentration cannot be determined unless n, the number of sites, is known.

When one-half of the sites are occupied, *i.e.*, $(Pr_t)/(Pr \cdot C) = 2$, then $K_{av.} = 1/(C)$. This value is 3.0×10^7 L. per M at 4° C. for both cases. Since the corresponding value for HSA is 0.5×10^4 L. per M (1), it is apparent that transcortin binds cortisol approximately 6,000 times more firmly than does albumin.

It would be advantageous to have an easier measure of the concentration of transcortin. Efforts to devise a measure have been thwarted by the presence of endogenous corticosteroids bound strongly enough to defy removal except under those circumstances which denature proteins. An alternative is to determine the amount of additional cortisol required to reduce the binding of a tracer amount of cortisol to an arbitrary level, say 50 per cent, or the decrease in binding produced by the addition of a fixed amount of cortisol, say 1 μg. The latter appears to be a more sensitive index as well as a more practical one. It should be realized, however, that this measures the difference between two variables, the level of transcortin and the level of corticosteroids.

Table IV compares such values for normal and

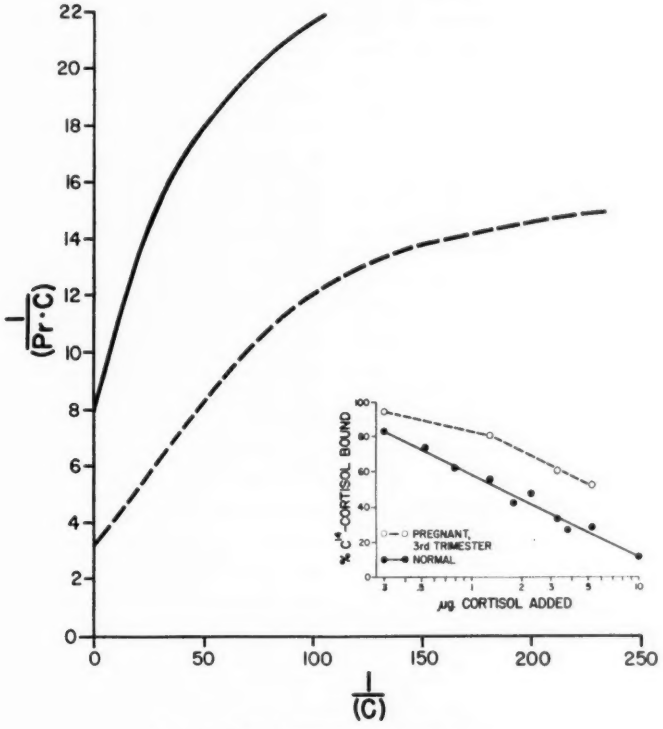

FIG. 1. THE BINDING CURVE OF CORTISOL

To 0.3 μg. of C^{14}-cortisol in 0.15 M saline was added 0 to 10 μg. of carrier cortisol. This was dialyzed against plasma diluted 1 : 5 with saline. The insert shows the data plotted as per cent C^{14}-cortisol bound *vs.* the amount of cortisol present. Each point is an average of determinations on three to six serums. Of more theoretical interest is the larger plot, which is calculated from the smaller one. The reciprocal of the concentration of bound cortisol is plotted against the reciprocal of the concentration of unbound cortisol, both expressed as L. per M × 60^{-6}. The solid and dashed lines denote plasmas obtained from normal and pregnant (third trimester) subjects, respectively.

TABLE IV

The decrease in binding of 0.3 μg. of C^{14}-cortisol upon the addition of 1.0 μg. of cortisol *

State	No. of subjects	% Bound before 1 μg.	Decrease in % bound after 1 μg.
Normal	6	82 ± 11.5†	26 ± 6.0
Pregnancy, third trimester	12	96 ± 2.0	13 ± 3.7
Pregnancy, second trimester	9	93 ± 1.6	20 ± 3.6
Pregnancy, first trimester	3	87 ± 5.5	21 ± 1.7

* Dialysis of plasma diluted 1:5 with saline against either saline or 1 per cent HSA.
† Average ± standard deviation.

for pregnant subjects in each trimester. As would be expected from Figure 1, plasma from pregnant women showed a progressively smaller decrease in binding as pregnancy progressed than did those from nonpregnant women. Since the corticosteroid level in the plasma of pregnant women is known to increase with the duration of pregnancy (11), we interpret this finding as an indication of increased transcortin levels during this period of pregnancy.

Because transcortin exhibits electrophoretic mobility, does not dialyze, and generally exhibits the characteristics expected of a protein, it has been assumed that it is a protein. As an additional check on the validity of this assumption, a sample of partially purified transcortin was digested overnight at 37° C. with trypsin at pH 7.5. The ability of transcortin to bind cortisol was completely lost.

The preceding dialysis was performed in the cold room. To answer questions regarding the

TABLE V

Effect of temperature on the binding ability and stability of transcortin *

Procedure	% Bound Expt. 1	Expt. 2	Expt. 3
Equilibrium dialysis at 4° C.	85	90	
Equilibrium dialysis at 37° C.	61	73	
Stand at 37° C. for 72 hrs.; then equilibrium dialysis at 4° C.	87	88	
Stand at 37° C. for 72 hrs.; then cortisol added and equilibrium dialysis at 4° C.	87	87	92
Stand at 4° C. for 72 hrs.; then cortisol added and equilibrium dialysis at 4° C.	86		94

* Two ml. of normal plasma diluted to 10 ml. with saline dialyzed against 30 ml. of 0.15 M NaCl and 0.3 μg. of C^{14}-cortisol.

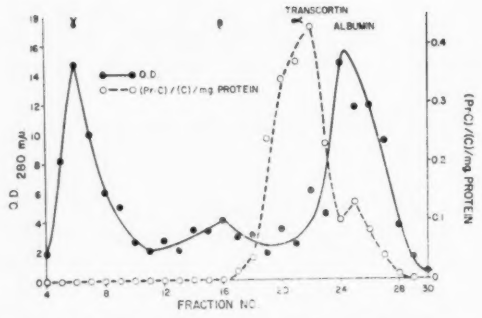

FIG. 2. CONTINUOUS FLOW PAPER ELECTROPHORESIS OF 25 ML. OF NORMAL HUMAN PLASMA IN 0.02 M BARBITAL BUFFER, PH 8.6, 660 VOLTS, *CA.* 50 MA.

The right-hand ordinate is the ratio of bound to unbound cortisol per mg. of protein.

effect of temperature on binding and stability of transcortin, the experiments outlined in Table V were performed on normal plasma. Elevation of the temperature produced a definite decrease in the amount of binding of cortisol, indicating a positive enthalpy of binding. This effect was not observed in the binding of cortisol and other steroids to human serum albumin (12). Transcortin is stable *in vitro* at 37° C. for three days, for no decrease in binding was observed upon return of the system to 4° C. as compared to the original determination or to a system which had remained at 4° C. throughout the experiment. Similar experiments on plasma from pregnant subjects led to the same conclusion.

Pooled blood-bank plasmas, collected in acid-citrate-dextrose (ACD) solution and stored in a refrigerator at 4° C., for various ages (fresh to four weeks old) showed essentially the same binding capacity. Freezing of these plasmas, or any other plasma, at −10°C. for several weeks did not affect their binding capacity for cortisol.

Continuous flow paper electrophoresis effects a considerable purification of transcortin and demonstrates that it is quite acidic. In Figure 2 are shown the results of electrophoresis of normal serum in barbital buffer at pH 8.6. The transcortin has migrated as an α-globulin. In Figure 3 is shown the result of electrophoresis of normal serum in acetate buffer at pH 5.2. It should be noted that the serum in this case was applied in the center instead of the left quarter point. The β- and γ-globulins have moved toward the cathode

while the α-globulins, albumin and transcortin have moved toward the anode. Transcortin has the mobility of an α-globulin. This result is in accord with that of Daughaday (5).

Fractions 22 through 26 inclusive (Figure 3) were pooled after withdrawal of one-third of the volume of each tube for determination of binding. After dialysis against two changes of distilled water to remove buffer, lyophilization yielded 10 mg. of dry residue, indicating a 150-fold protein concentration over the original plasma. Further purification and characterization are being continued.

Transcortin is also present in varying degrees in the blood of animals (Table VI). The dog appears to have very little transcortin while rabbits, guinea pigs, rats and perhaps alligators have substantial concentrations.

DISCUSSION

It is apparent from the data of our experiments and from those published by Daughaday (4) that human and some animal plasmas contain a protein (or proteins) with a great avidity for the binding of cortisol. We have named this protein transcortin with the full realization that it may actually constitute a system of several proteins rather than a single one and that under abnormal conditions the changes in the binding capacity for cortisol may not be related necessarily to changes in transcortin concentrations. Nevertheless, we believe that the name simplifies communication

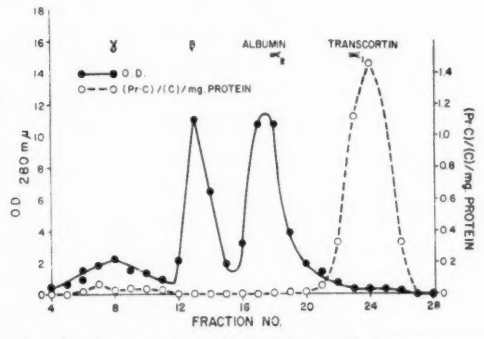

FIG. 3. CONTINUOUS FLOW PAPER ELECTROPHORESIS OF 25 ML. OF NORMAL HUMAN PLASMA IN 0.02 ACETATE BUFFER, pH 5.2, 800 VOLTS, 40 TO 60 MA.

The right-hand ordinate is the ratio of bound to unbound cortisol per mg. of protein.

TABLE VI

Binding of cortisol to animal plasma *

Amount of carrier	Rabbit	Dog	Guinea pig	Rat	Alligator
0 μg.	91†	39	83	86‡	93
2	60	20	70	63	
5	48	12	61	49	
10	36		52	40	
20		8	38		

* Plasma diluted 1:5 dialyzed against saline.
† Corticosterone, 82 per cent.
‡ Corticosterone, 75 per cent.

and does not commit any particular protein (glycoprotein, lipoprotein, and so forth) as being the cortisol-binding protein until rigid proof of the identity is established. In agreement with Daughaday (5) we believe transcortin to be associated with the α-globulin area in the electrophoretic separations.

Since no specific method exists for determining the concentration of transcortin, recourse was made in our experiments, as well as in those of Daughaday (4, 5), to the ability of human plasma to bind cortisol *in vitro* during equilibrium dialysis. The estimate of the transcortin binding capacity under these conditions is only an approximation. The reasons for this statement are as follows. Although transcortin binds cortisol most avidly, it appears that it can also bind corticosterone, 11β-hydroxyandrostenedione and other steroids to a lesser extent. In addition, it would be difficult to deny, until definite evidence is presented, that transcortin does not bind conjugates and metabolites, both unknown and known, of many other steroids. Since the determination of the concentration of these various steroid metabolites is at the moment impossible, it is apparent why the determination of the binding capacity of human plasma for cortisol remains an approximation. Nevertheless, the reproducibility and constancy of the binding capacity of transcortin under the conditions of our experiments offer at present the best method for the quantitative estimation of transcortin concentration.

The results of the competition experiments are in accord with the hypothesis that there are two types of sites at which corticosterone and cortisol may be bound. For example, at one type of site cortisol may be bound strongly and corticosterone weakly so that the former would have a greater

inhibiting effect on the binding of C^{14}-cortisol. At the second type of site both steroids would then be bound with an intermediate strength; otherwise, the first type of site would bind nearly all the cortisol.

Using undiluted plasma, Daughaday (13) failed to show any differences between the binding of normal and pregnant subjects. On the other hand, with the use of diluted plasma and the addition of carrier cortisol we were able to show a definitely increased transcortin capacity in the plasmas of pregnant women during the third trimester. Daughaday's failure (13) to show differences between normal and pregnant plasmas was due to the high equilibrium constant of transcortin combined with the use of a high protein concentration. The increased levels of transcortin during the third trimester of pregnancy are even more pronounced in light of the possibility that the greatly increased levels of other steroids (progesterone and its derivatives, corticosteroids, and so forth) infringe on the capacity of transcortin to bind the added labeled and carrier cortisol. The identity of equilibrium constants for both normal and pregnant plasma indicates that no qualitative changes in transcortin have occurred as a result of pregnancy. In agreement with Daughaday (4) we believe that at so-called physiological concentrations of cortisol in plasma it is bound preponderantly by transcortin. It should be pointed out, however, that equilibrium must exist between transcortin-bound cortisol, cortisol present in solution and albumin-bound cortisol. Thus transcortin, like albumin, would function in the transport of cortisol through the vascular system.

Although some of the concepts to be presented are merely heuristic, we believe that they can be of help in furthering the identification and study of the significance of transcortin. We propose that transcortin-bound cortisol may be for all practical purposes biologically inactive, whereas that cortisol which is not bound to transcortin is physiologically active. Thus, when the cortisol concentration exceeds the transcortin capacity, marked physiological effects will appear. This is seen following the administration of pharmacological doses of cortisone or cortisol or during increased adrenocortical activity (stress, adrenocorticotropic hormone [ACTH] administration). The lack of real evidence of hyperadrenocorticism during pregnancy, in spite of the elevated concentrations of cortisol, can best be explained on the basis of increased transcortin concentrations. On the other hand, it is possible that in some patients with Cushing's syndrome having normal levels of corticosteroids in the plasma, the disease may be due to the decreased concentrations of transcortin, resulting in an increased concentration of nontranscortin-bound cortisol with resultant increased physiological effects. The levels of transcortin in this and in other conditions in which adrenocortical function have been implicated as a factor are now under study in our laboratory. It will also be interesting to ascertain the role of transcortin in the regulation of ACTH secretion and whether transcortin-bound cortisol is metabolized in a fashion similar to that which is not bound to transcortin. These studies await the purification and ultimate isolation of transcortin, so that it can be administered with or without cortisol.

Our failure to demonstrate a protein-binding system, akin to transcortin, for the estrogens corroborates the findings of Bischoff and associates (9, 10) and indicates the lack of existence of a special protein for the binding of these steroids as postulated by Roberts and Szego (8). These authors indicated that Fraction III-0 of Cohn was mainly responsible for the binding of the estrogens. Our data and those of Bischoff and co-workers (9, 10) point towards albumin as the protein system primarily responsible for the binding and transport of estrogens. Indeed, the data of the present study indicates that albumin plays a paramount role in the binding, and hence probably transport, of such steroids as progesterone, testosterone and some of the other androgens.

SUMMARY

There is in human plasma of normal and pregnant subjects, as well as that of a number of animals, a protein having a high affinity for cortisol ($K = 3.0 \times 10^7$ L. per M) but which is present in low concentrations (1.3×10^{-7} M per L.). We propose to call this protein transcortin. Competition experiments showed that cortisol and corticosterone are bound at two types of sites. At one, cortisol is definitely bound more strongly

than corticosterone, while at the second, they are approximately equal in strength of binding. Estrogens, androgens and progesterone are bound weakly or not at all by transcortin. A method of measuring transcortin concentration is described. Using this method it was found that transcortin levels increase during pregnancy. A speculative hypothesis was advanced to explain the physiological significance of transcortin. Basic to this hypothesis is the assumption that corticosteroids bound to transcortin are biologically inactive. The concentration of corticosteroids in the plasma must, therefore, exceed the effective concentration of transcortin before physiological, and especially pharmacological, effects of the corticosteroids become evident. Transcortin is stable for at least one week in plasma or in dilute solution at either 4 or 37° C. It is destroyed by incubation with trypsin. Continuous flow paper electrophoresis at pH 5.2 separates transcortin from the bulk of the plasma proteins, effecting a 150-fold purification. It is believed to be an α-globulin.

ACKNOWLEDGMENTS

We wish to acknowledge the cooperation of Dr. Robert Patterson of the Buffalo Medical Group, who supplied us with plasma from pregnant subjects; the generous donation of human serum albumin by the American Red Cross and of C^{14}-labeled corticosteroids by the Endocrinology Study Section of the National Institute of Health; and the valuable technical assistance of Miss Jean Rae Schubarg, Mr. Lawrence Beecher and Mr. Elek Karsay.

ADDENDUM

After submission of our manuscript an interesting article by Dr. Ian E. Bush (Ciba Foundation Colloquia on Endocrinology, 1957, **11**, 263) on "The Physicochemical State of Cortisol in Blood" came to our attention. This article describes experiments pointing to the existence of a cortisol-binding protein which becomes saturated at the upper level of the normal range for plasma cortisol concentrations.

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BODY IRON EXCHANGE IN MAN*

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The present studies were undertaken to measure total body iron turnover in man. The assumptions were made: *a*) that the adult is in iron equilibrium, and *b*) that it is possible by the intravenous injection of iron to label body iron uniformly to the extent that blood sampling will reflect iron exchange. Such studies had previously been carried out in animals and the premise was shown to be generally valid (1). This report indicates the approximate period of time required for uniform labeling of miscible iron, and characterizes the total iron turnover in normal men, in nonmenstruating and in menstruating women.

MATERIALS AND METHODS

Patients in the Northern State Hospital were selected for study because of their good health and normal hematologic findings, and absence of history of abnormal blood loss or anemia. They were divided into three groups. Group I was composed of 6 adult men, Group II of 12 adult nonmenstruating women and Group III of 6 adult menstruating women. Pertinent hematologic data are summarized in Table I.

Hematocrit, plasma iron and reticulocyte determinations were performed initially and at the end of the study on venous blood samples from these patients. The plasma iron method employed was that of Bothwell and Mallett (2). Radioiron was injected intravenously as Fe^{59} citrate in dosage of 100 μc. (60 μg. of iron) per patient. Samples were drawn on the second month after injection and at 4 month intervals thereafter over a period of from 46 to 54 months. On each occasion the red cell hematocrit was determined, and duplicate samples of blood were pipetted with a calibrated pipette for radioactive analysis. At the end of the study all samples were wet-ashed, precipitated, electroplated and counted as previously described (3).

In calculation of data, average figures of red cell activity for each group were plotted on semilog paper. Employing these figures after the first year, a line was fitted by the method of least squares and extrapolated to zero time. The slope of this line appeared constant over a three year period and was taken to represent external loss of iron. Turnover rate was then calculated from this slope according to the formula:

$$\frac{\text{Turnover rate}}{\text{(per cent per year)}} = \frac{0.693}{T\frac{1}{2}\text{ (in years)}} \times 100 \qquad (1).$$

Red cell mass was estimated on the basis of an assumed blood volume of 64 ml. per Kg. and the determined red cell hematocrit. The factor 1.1 was used to convert red cell volume (ml.) to iron content (mg.). The formula employed was:

$$\text{Red cell iron (mg.)} = \text{body weight (Kg.)} \times \text{hematocrit} \times 64 \times 1.1.$$

The estimation of miscible tissue iron was derived from two sources: *a*) The initial distribution of radioiron in the normal subject at 60 days was assumed on the basis of other studies [1] to be 10 per cent in tissues other than circulating red cells. Thus, early tissue localization of iron would be represented by the formula:

$$\text{Red cell iron (mg.)} \times \frac{10}{90}.$$

b) There was an indication from the plot of red cell activity that the fall during the first year was excessive. The magnitude of this initial component was obtained by extrapolating the latter slope to zero time and subtracting this value from 100 per cent. This was interpreted as due to mixing of radioiron with tissue iron and its amount in mg. of iron was calculated from:

$$\text{Red cell iron (mg.)} \times \frac{\text{initial component (per cent)}}{100\text{ per cent} - \text{initial component (per cent)}}.$$

It was assumed that a uniform specific activity was attained in the course of these studies between red cell and miscible tissue iron. Total miscible body iron then was represented by the addition of these two fractions of labeled tissue iron to red cell iron. Turnover rate was applied to this total miscible iron figure in the calculation of daily iron absorption and excretion. It was assumed that the subjects studied were in a steady state; that is, that iron absorption and excretion were equal over the period of study.

RESULTS

Radioiron data obtained in individual subjects are shown in Figure 1, and average data in the

* This investigation was supported by a research grant from the Atomic Energy Commission [Contract AT-(45-1)-218] and United States Public Health Service Grant No. H-2994.

[1] This is based on the determination of the amount of radioiron in circulation in five normal subjects at 60 days, employing Cr^{51} to estimate red cell mass (4).

TABLE I

Hematological data on subjects

Subject	Age	Sex	Weight	RBC iron	Hematocrit			Reticulocytes		Plasma iron		Blood volume
					Initial	Average	Final	Initial	Final	Initial	Final	
			Kg.	*mg.*	%	%	%	%	%	*μg.* %		*ml.*
Group I (Men)												
J. A.	82	M	65	2,015	42	44	44	0.4	0.7	88	116	4,160
P. A.	84	M	64	1,805	39	40	39	1.4	1.5	73	98	4,100
A. B.	61	M	59	2,040	50	49	47	0.1	0.1	112	66	3,780
P. B.	70	M	86	2,895	46	47	49	1.9	1.1	56	156	5,600
O. O.	64	M	57	1,685	42	42	41	1.0	0.4	112	75	3,650
T. O.	57	M	63	2,080	46	47	50	0.8	0.6	68	100	4,030
Average values			66	2,085	44	45	45	0.9	0.7	85	102	4,220
Group II (Nonmenstruating women)												
J. A.	75	F	57	1,685	42	42	44	0.6	0.9	82	82	3,650
S. A.	77	F	77	2,385	43	44	45	0.8	0.3	66	67	4,930
M. B.	76	F	57	1,765	44	44	46	0.1	0.5		110	3,650
A. B.	67	F	43	1,210	38	40	41	0.3	0.5		59	2,750
F. B.	70	F	52	1,610	44	44		1.3		100		3,330
I. B.	56	F	62	1,830	42	42	42	0.3	0.9	100	77	3,970
E. B.	63	F	68	1,965	38	41	43	0.7	0.4		108	4,350
A. Bo.	74	F	58	1,875	47	46	45	1.7	1.0	70	128	3,710
H. G.	68	F	73	2,155	43	42	42	0.3	0.8	106		4,670
F. G.	64	F	50	1,690	46	48		0.2				3,200
B. S.	50	F	64	2,115	48	47	47	0.6	1.0	75	105	4,100
A. G.	49	F	45	1,300	40	41	40	0.6	0.9	39	75	2,800
Average values			59	1,799	43	43	44	0.6	0.7	80	90	3,759
Group III (Menstruating women)												
D. C.	37	F	55	1,590	43	41	41	1.5	0.4	154	177	3,520
E. C.	39	F	67	2,170	46	46	42	1.2	1.1	75	261	4,290
A. O.	40	F	59	1,785	44	43	40	0.8	1.2	89	122	3,775
E. P.	44	F	57	1,605	39	40		0.7		115	130	3,648
E. B.	33	F	59	1,910	45	46	46	1.7	1.0	97	128	3,775
L. R.	32	F	66	1,950	41	42	41	1.1	1.2	81	97	4,225
Average values			61	1,835	43	43	42	1.2	1.0	102	153	3,872

three groups are shown in Figures 2a, b and c. In all instances the blood value at two months after initial injection was taken to represent 100 per cent and subsequent values are plotted as a fraction of this value. An excessive fall was observed during the first year of 15 per cent in men and 9 per cent in women. The slope of decreasing red cell radioactivity over the following 3 years in these three groups indicated a half-life of 8.3 years in men, 6.4 in nonmenstruating women and 3.4 years in menstruating women. This is equivalent to yearly decreases in red cell radioactivity of 8.3, 10.8 and 20.1 per cent in these three groups.

The miscible tissue (or non-red cell) iron in the three groups, derived from an assumed 10 per cent tissue distribution at the time of injection and from the excessive fall in activity during the first year, was 600 mg. in men and 380 mg. in women (Table II). Daily turnover of total miscible iron was calculated to be 0.61 mg. per day in men, 0.64 mg. per day in nonmenstruating women and 1.22 mg. in menstruating women.

DISCUSSION

Assuming the normal adult to be in iron balance, it should be possible to determine iron turnover by measuring quantitatively either absorption or excretion. There are serious limitations in the use of absorption measurements. Chemical balance studies have the advantage of measuring the total intake of iron in its dietary form, but are of insufficient precision. Isotopes of iron, which have the advantage of more accurate quantitation, do not allow one to administer iron in its various forms as they occur in food. However, studies

done in which radioiron was added to food (5) or in which various foodstuffs were biosynthetically labeled (6–8) suggest an absorption of about 5 per cent or 0.75 mg. per day from a normal diet.

A second approach to the quantitation of iron balance is the measurement of iron loss. Chemical measurements are not feasible, but losses of radioiron, measured by periodic collections of urine, feces and sweat over a period of 140 days have been reported by Dubach, Moore and Callender (9). Daily losses of 0.38 to 0.52 mg. of iron were calculated in three male and one female subjects.

In the present study body iron turnover was determined by sampling red cell iron over a period of three to five years. Calculations of the daily turnover of red cell iron after the first year gave values of 0.47, 0.53 and 1.0 mg. per Kg. in men, nonmenstruating and menstruating women, respectively. After allowance was made for mixing with other body iron, the calculated daily exchange of body iron was 0.61, 0.64 and 1.22 mg. in three groups of subjects (Table II). Since the possibility of further gradual mixing of radioiron in circulation with tissue iron cannot be excluded, it should be emphasized that these calculated rates of iron excretion represent maximal values.

Dubach and co-workers (9) obtained evidence that iron losses were decreased in iron deficiency and increased with hemolytic anemia. Similar results indicating a direct correlation between amount of body iron and excretory losses were obtained by us in the mouse (1). It is known as well that both the state of iron stores and the rate of erythropoiesis influence absorption. Thus the data here which apply only to iron turnover in the normal subject may well be appreciably modified in pathologic states.

Of additional interest was the evaluation of miscible tissue stores by this technique. In all three groups the time required to reach a constant

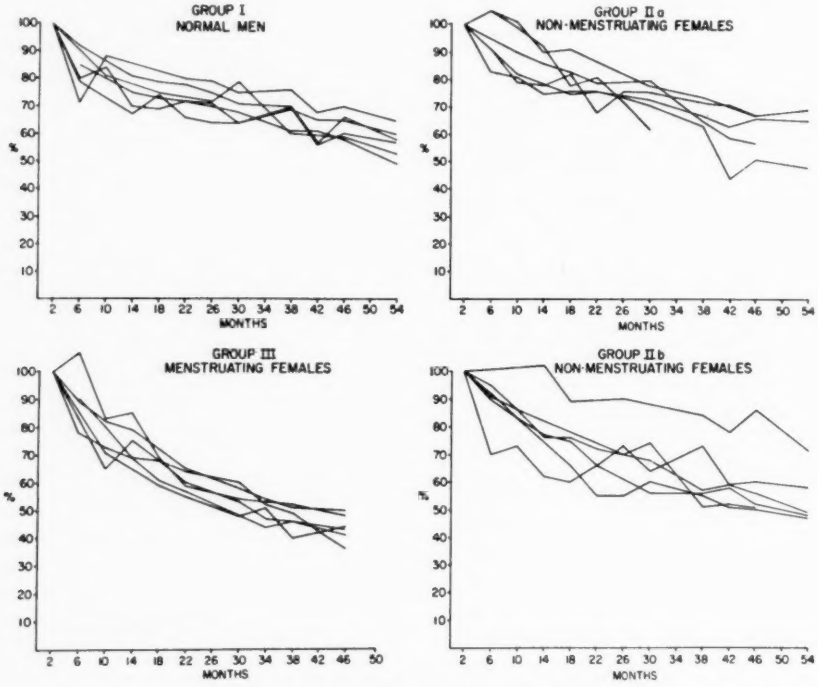

FIG. 1. Fe^{55} ACTIVITY OF CIRCULATING BLOOD

Levels of red cell activity over a period of 54 months are plotted. The value at 60 days in each instance is taken as 100 per cent.

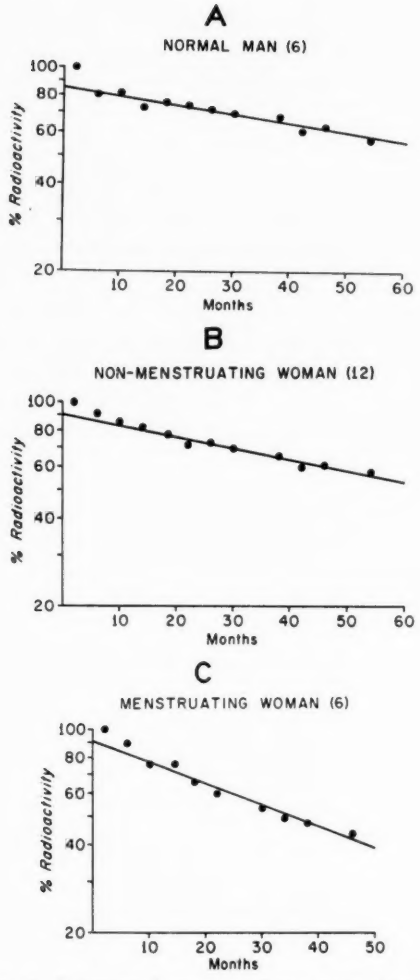

FIG. 2. RADIOIRON LOSS FROM CIRCULATING ERYTHROCYTES OF NORMAL MAN (A, SIX SUBJECTS), NONMENSTRUATING WOMAN (B, 12 SUBJECTS) AND MENSTRUATING WOMAN (C, SIX SUBJECTS)

Average values for each group of subjects are plotted. The line expresses the slope of fall in red cell radioactivity after the first year.

daily loss was approximately one year. The average size of body stores calculated from the size of this original mixing component, and with allowance for an initial tissue iron labeling by about 10 per cent of the injected activity, was from 377 to 600 mg. The figure of 600 in men is about half of that previously shown to be available for hemoglobin synthesis by phlebotomy experiments (10). Thus it seems likely that only a portion of storage iron (ferritin and hemosiderin) is miscible in the normal individuals over prolonged periods, despite the fact that it may all be mobilized from tissues if needed for red cell production.

SUMMARY

Red cell iron turnover as measured by the radioactivity of the circulating red cell iron over a four year period was found to represent 0.47 and 0.53 mg. per day in a group of 6 men and 12 nonmenstruating women. The amount of miscible tissue iron was identified by an exaggerated fall in the first year. This miscible pool is appreciably smaller than estimates of nonhemoglobin body iron. Iron turnover of total miscible body iron is calculated to be 0.61 mg. per day in men, 0.64 mg. per day in nonmenstruating women and 1.22 mg. per day in menstruating women.

ACKNOWLEDGMENTS

We would like to thank Dr. Charles H. Jones, Superintendent, for his cooperation, and also the staff of the Northern State Hospital, Sedro-Woolley, Wash., for making these studies possible.

We would like to express our appreciation to Dr. Lars Garby for his assistance in analyzing the data, and for his helpful criticisms.

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TABLE II

Iron metabolism of the experimental subjects

	Group	Red cell iron	Miscible tissue iron	Total miscible iron pool	Pool turnover		Daily absorption or excretion
		mg.	*mg.*	*mg.*	*%/year*	*mg./year*	*mg.*
I	Men	2,085	600	2,685	8.3 ± 1.1	223	0.61 ± 0.08
II	Nonmenstruating women	1,799	377	2,176	10.8 ± 0.8	235	0.64 ± 0.05
III	Menstruating women	1,835	385	2,220	20.1 ± 1.7	446	1.22 ± 0.11

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ESOPHAGEAL CATHETERIZATION STUDIES. I. THE MECHANISM OF SWALLOWING IN NORMAL SUBJECTS WITH PARTICULAR REFERENCE TO THE VESTIBULE (ESOPHAGO-GASTRIC SPHINCTER) *

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Although studies of motility by means of an indwelling balloon have furthered understanding of the physiology of the esophagus, this method does not readily lend itself to the simultaneous measurements from multiple sites that are necessary for understanding the coordinate activities of deglutition (1–3). Several investigators were better able to study peristalsis in this organ by utilizing tandem systems with open-tipped catheters or transducers (4–10). Sanchez, Kramer and Ingelfinger first pointed out that the motor activity of the vestibule, or distal 2 to 5 cm. of esophagus, is different from that of the body (5). Since then there has been considerable discussion regarding the different motility patterns recorded from this area and how to interpret them (6, 9–11). A good method for the study of the vestibule should allow for sensitive, simultaneous recordings from closely approximated sites, be sufficiently comfortable to the subject to permit prolonged observations, and of course, yield reproducible results.

The purpose of this paper is to describe a technique which fulfills these criteria and to report on the results obtained in 15 observations of esophageal motility in 10 healthy adult subjects. The normal patterns described here will be compared (in subsequent communication) with those obtained in disease states and during drug administration.

METHODS AND MATERIALS

Equipment. The catheterization set consisted of six polyethylene tubes (Intramedic®, I.D. 0.055, O.D. 0.075, P.E. 2000)[1] cut in three foot lengths and mounted side by side. The catheters were sealed at their distal ends to a metal gastric bucket so that in effect they represented a single tube with six separate lumina. Lateral openings, 2 mm. in diameter, were made at fixed intervals from the gastric tip and marked with radiopaque thread (Figure 1).

The catheters were filled with water and pressure changes were transmitted through these columns to three Sanborn electromanometers and a four-channel Sanborn polyviso recorder. Details of the operation of the de-

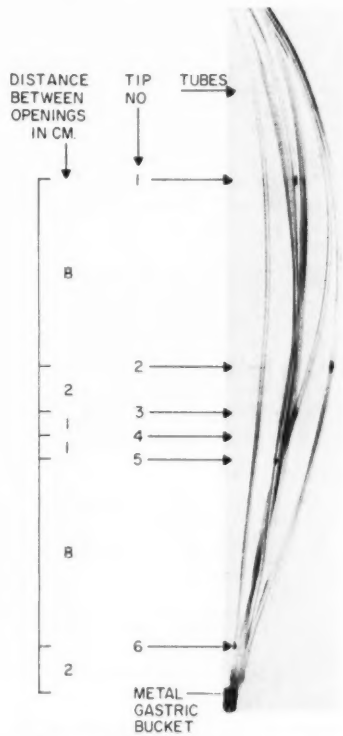

FIG. 1. ESOPHAGEAL CATHETER SET

* This study was supported by a grant-in-aid of the G. D. Searle and Company, Chicago, Ill., the Ida and William Rosenthal Foundation, and Research Grant RG 4460 from the National Institutes of Health, United States Public Health Service.

[1] Obtained from Clay-Adams, Inc., New York, N. Y.

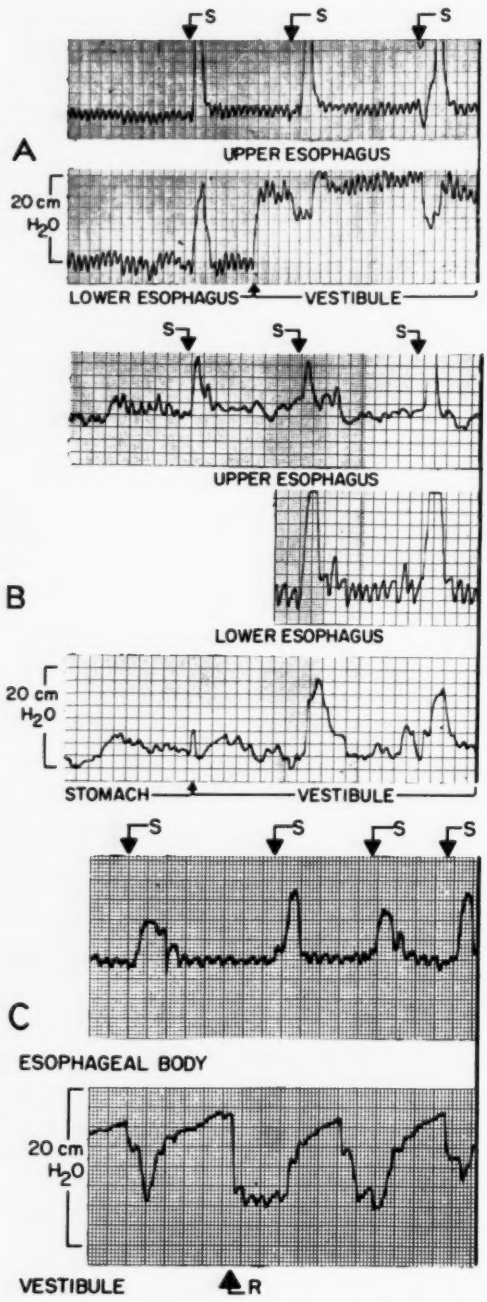

FIG. 2. EFFECT OF RESTING PRESSURE IN THE VESTIBULE ON MOTILITY DURING SWALLOWING

A. The initial portion of Record A shows a simultaneous recording from the upper and lower esophageal body. By switching the stopcock from the lower esophageal tip to a catheter located 2 cm. below, a vestibular pattern is recorded with a resting pressure about 20 cm. over the pressure in the esophageal body. Note that with this elevated resting pressure the entire response of this segment to swallowing is a sustained fall in pressure lasting 8 to 10 seconds.

S↓, swallow; ↑, switching of recording catheter to vestibule.

B. In Record B, the bottom tracing shows gastric motility. By switching the stopcock to a tip 2 cm. higher, a vestibular pattern is recorded. In this instance there is virtually no difference in resting pressure between the stomach and the vestibule. Note that during swallowing the major component of the vestibular response consists of a positive pressure wave.

C. In Record C, the initial portion of the vestibular record shows an elevated resting pressure and relaxation during swallowing. There is a spontaneous relaxation of resting pressure indicated by R, and at this lower resting pressure the response to swallowing consists of a positive pressure wave.

tecting and recording apparatus and of the connections between catheters and these instruments were previously described (12). The records were standardized at 20 cm. H_2O full scale with a paper speed of 1 mm. per second, or for higher pressures, at 40 cm. H_2O and 0.5 mm. per second.

The scale on the figures indicates magnitude of pressure change without reference to a zero pressure. This permits full use of the scale and depicts accurately the pressure gradients between segments, which are the determining factors in the movement of the bolus. The method also eliminates the additional error introduced by comparing measurements to atmospheric pressure. This is sometimes inaccurately referred to as "absolute pressure," but is in fact another method of expressing relative pressure but in reference to an extraneous variable—atmospheric pressure.

An important feature of the apparatus was the interposition of three 3-way stopcocks between the six catheters and the connections to the electromanometers (Figure 3). To each of these, two catheters were attached. By switching the stopcock connections, it was possible to select the desired sites without moving the catheters. Such switching introduced no significant difference in the accuracy of interpretation of pressure changes when this maneuver was confined to tips Nos. 3, 4 and 5, which were positioned 1 to 2 cm. apart. In this manner, pressure in the vestibule relative to that in the stomach or in the body of the esophagus could be determined at any time by changing from the recording tip in the vestibule to one in the body above or one in the stomach below. Also, switching aided in locating the terminal segment. Vestibular pressure relative to gastric or body pressure was frequently ascertained before the effect of swallowing was studied (Figure 2).

To record the pressure exerted by the closed organ, a modification of the pump described by Lorber and Shay

(13) and Davidson, Sleisenger, Almy and Levine (12) was employed. This pump maintained a flow of water through the catheters at the rate of 3 ml. per hour (Figure 3). The effect of this flow upon the form of the pressure tracing recorded from the vestibule was tested in five experiments with four subjects, with two catheters placed at the same level. No significant differences were observed on comparison of records obtained with and without the pump except for more frequent loss of the usual phasic activity associated with respiration when the pump was not employed. This indicates that the replacement by the pump of the small amounts of fluid being lost at the dependent tip of the catheter diminishes the possibility of damping in the pressure transmission, because of air bubbles.

The relationship of the pump to the responsiveness of the pressure detecting system under conditions when the recording tip is occluded, as would exist when a sphincter is closed around the catheter, is seen in Figure 4. The figure illustrates the effect on the pressure record when the tip of a single catheter of the type used in the studies was manually occluded with the pump turned on and off, while the catheter was connected to the pump and electromanometer. During simultaneous recording at the same level, no change in the form of the record was produced in the second catheter when the pump was turned on or off in the first catheter. This indicates that these pressure fluctuations are probably not the result of pumping water into the small closed space of the vestibular lumen.

When the diameter of our recording catheter was increased and it was made more rigid by fastening the tubes together with an elastic band, pressure changes were diminished and fluctuations in the resting pressure of the sphincter were minimized.

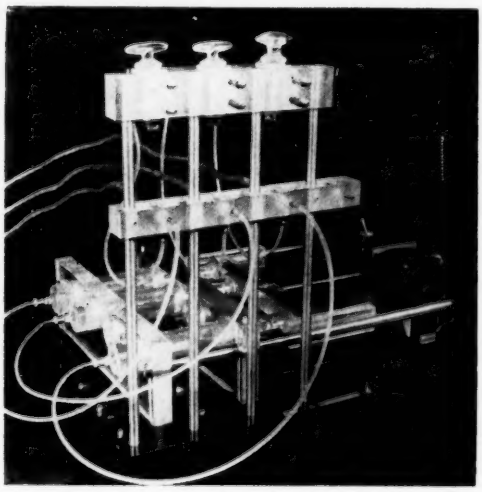

FIG. 3A. PUMP ASSEMBLY WITH THREE 3-WAY STOPCOCKS FOR ATTACHMENT OF THE ESOPHAGEAL CATHETER SET

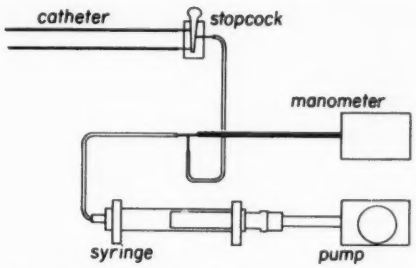

FIG. 3B. DIAGRAMMATIC REPRESENTATION OF FIGURE 3A

In an effort to determine if any component of the wave forms was caused by the flow of water past the tips during deglutition, a special set of catheters was prepared. Three polyethylene catheters were attached together, one with a lateral opening, one with an end opening directed caudad, and one with an end opening cephaled. All openings were at the same level. Motility tracings demonstrated that the flow was too slow to have any detectable influence on the pressure waves and that the recording from the lateral opening was identical to those from the catheters with end openings.

A pneumograph placed around the chest of the subject and connected to the fourth channel served as a check on the effect of respiration, coughing, talking and so forth upon the recording.

Procedure. In the fasting state and without anesthesia or premedication, the subject swallowed the catheters which were positioned fluoroscopically so that the gastric tip (No. 6) was in the body of the stomach and No. 5 opening was about 2 cm. below the diaphragm. Because the motility of this segment was found to be different from that of the stomach, it was considered to be

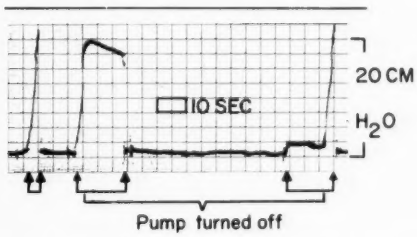

FIG. 4. EFFECT OF SUDDEN OCCLUSION OF CATHETER OPENING ON PRESSURE RECORD

Periods of catheter occlusion are marked by arrows. Note that when catheter is occluded with pump on, there is marked rise in pressure and prompt return to baseline with release of occlusion. During occlusion the pressure falls slowly when pump is turned off (second complex). Later, with pump still off, occlusion produced only slight rise in pressure; however, when the pump was again turned on, a sharp rise in pressure is recorded (final complex).

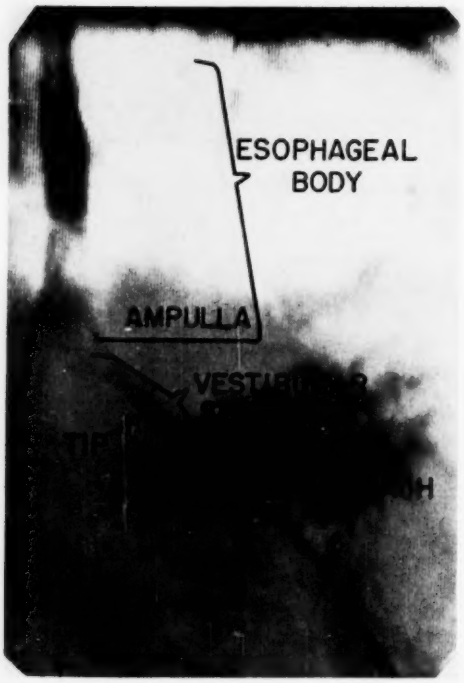

FIG. 5A. SPOT FILM ILLUSTRATING LOCALIZATION OF TIP INFRADIAPHRAGMATICALLY IN STUDIES OF THE TERMINAL SEGMENT OF ESOPHAGUS

esophagus. Miokon®[2] was injected into the catheters and their positions demonstrated fluoroscopically and by spot films (Figure 5).

The respiratory tracing also aided in localizing the openings. Above the diaphragm, inspiration caused a decrease in intraesophageal pressure and was registered as a downward deflection of the recording needle. Conversely, normal inspiration caused the infradiaphragmatic catheter to record an upward deflection as did gentle manual pressure over the stomach. A recording tip at the level of the diaphragm, however, described a respiratory record of lower amplitude which was out of phase with both of these. In the infradiaphragmatic esophagus, a deep breath produced an immediate upward deflection followed by a rapid downward deflection as the diaphragm passed caudad to the catheter tip (Figure 6).

Although preliminary observations indicated that no appreciable differences could be observed when patients sat or when they reclined, the studies were performed with the patient in the sitting position because this is the usual body position during swallowing. Continuous aspiration of saliva with a catheter situated beyond the cricopharyngeus reduced involuntary swallowing, but during experimental observations this was not carried out since the effect of involuntary as well as of voluntary swallowing was of interest. The effects of coughing, retching, belching, swallowing saliva and deep inspiration were noted on the tracings.

To study deglutition, the subject was given 10 ml. of water to be held in the mouth and swallowed on command. Continuous pressure measurements were made over periods of one-half to three hours. In the analyses of all records, the presence or absence of each component was noted as were the duration and maximum amplitude of the complex.

[2] Miokon® was generously supplied by W. R. Armstrong, Mallinckrodt Chemical Works, St. Louis, Mo.

RESULTS

Both during swallowing and in the resting state there are characteristic wave forms of the upper eight-ninths (body) and lower one-ninth (vestibule) of the esophagus (Figure 7).

Resting state

In the resting state only respiratory movements are discernible in the body. The vestibular segment may exhibit a relatively high resting pressure (Figures 2A and 8B) or a changing tone (Figures 2C and 8C). Pressure usually varies between 10 and 40 cm. H_2O but may reach even 100 cm. H_2O over resting gastric pressure. By noting the distance between the tips recording this characteristic pattern, usually Nos. 3, 4 and 5 (Figures 1 and 7), the length of this segment was found to vary from 2 to 5 cm. with an average in five normal subjects roughly estimated at 2.7 cm.

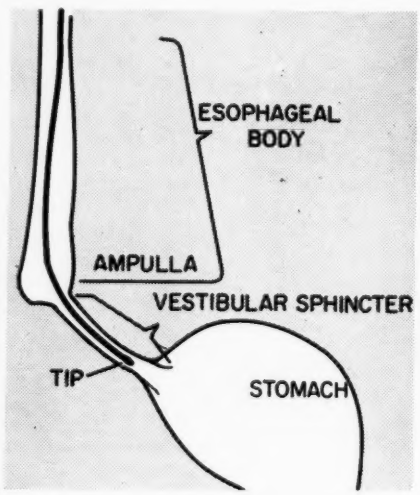

FIG. 5B. DIAGRAMMATIC REPRESENTATION OF FIGURE 5A

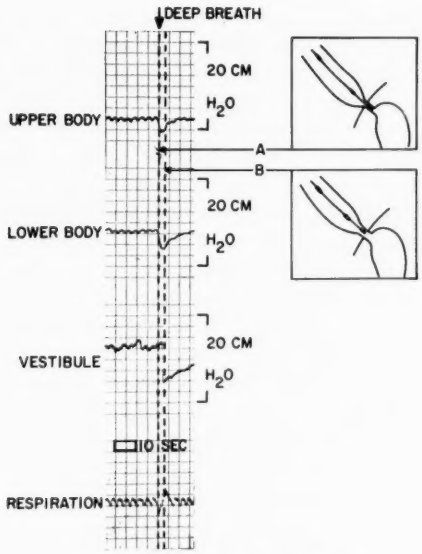

FIG. 6. EFFECT OF A DEEP BREATH

The respiratory record aids in localizing the recording tips. The vestibule does not register fall in pressure until the diaphragm has passed caudad to the recording tip during deep inspiration.

Swallowing

The usual wave form of the esophageal body during swallowing has five recognizable components (Figure 7). Component 1 occurs during the sipping of a mouthful of water; if, however, the subject retains liquid in his mouth prior to swallowing, this wave will not appear. Under the conditions used in these studies, it appears that this wave is caused by the involuntary inspiration which occurs with sipping. Our interpretation is in agreement with others that Wave 2 is related to the contraction of the pharyngeal muscles. It is transmitted to the esophageal body tracing via the bolus. It seems probable that Component 3 represents a summation of the decline in Wave 2 and the increase in pressure of Wave 4 and thus appears as a plateau. The major component, Wave 4, represents peristaltic contraction and usually has a smooth, bell-shaped contour. It travels at the average rate of 2.5 cm. per second (1.9 to 5.5 cm. per second) and, with the recording system used, attains a pressure of 15 to 20 cm. water. Its duration is 8 to 10 seconds. Occasionally it reaches a height of 25 cm. water and, rarely, 40 cm., and may last as long as 18 seconds. Deflection 5 represents the effect of respiration. This type of deflection, therefore, may occur in any part of the complex.

With the onset of deglutition, there is a brief relaxation of the vestibule, the degree of pressure drop being in part dependent upon the resting pressure (Figures 2 and 8). Thus, in some instances the relaxation was not apparent when the difference between resting pressure in the vestibule and stomach was relatively small even though the position of the recording tip in the vestibule was established by both manometric and radiologic criteria (Figures 2B and 8B). Fluoroscopic examination will show that some material may be passed into the stomach at this time. This probably represents a mechanism for emptying the esophagus before the bolus reaches the segment. As the peristaltic wave travels down the body,

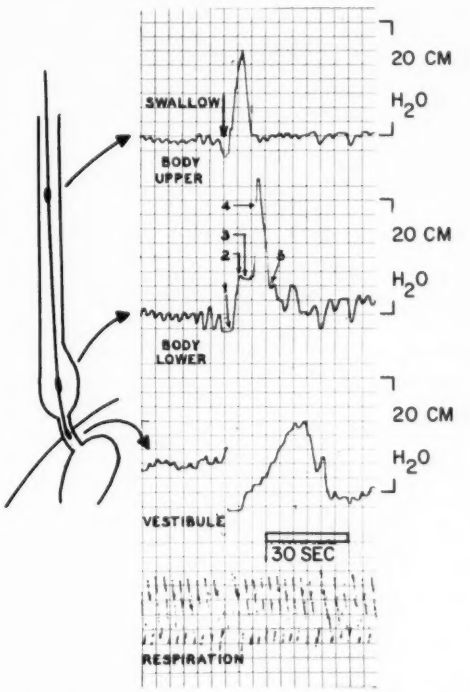

FIG. 7. PRESSURE CHANGES IN NORMAL SWALLOWING

This is a multiple tip recording demonstrating pressure changes in the body and vestibule during the swallowing of water. Components 1 through 5 of complex are described in text.

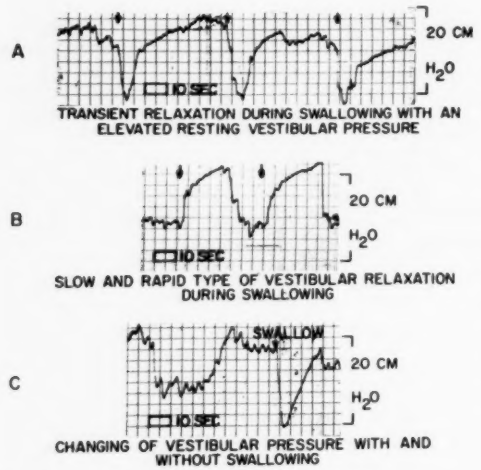

FIG. 8. DIFFERENT TYPES OF ACTIVITY OF THE VESTIBULAR SPHINCTER

These tracings were recorded from three different normal individuals, and illustrate the types of pattern which may be observed in any normal subject. Assessment of vestibular pressure before swallows as "high" or "low" is in reference to either pressure in the gastric fundus or esophageal body (Figure 2). Scale indicates magnitude of pressure change. Swallowing occurs at arrows only.

A. With a high resting vestibular pressure, swallowing frequently produces a single relaxation with a return to the elevated baseline.

B. Note that there is no initial relaxation with the onset of each of two swallows. This is observed when the resting pressure at the time is only slightly more than gastric pressure. The ensuing pressure rise may be terminated slowly (first swallow) or rapidly (second swallow).

C. Spontaneous changes in the resting vestibular pressure are illustrated. The relaxation which occurred with swallowing is indicated by the arrow.

the pressure in the vestibule gradually rises to 5 to 20 cm. water and lasts from 5 to 30 seconds. This rise is terminated with a sudden drop in pressure in 80 per cent of studies. In the remaining 20 per cent, especially when the vestibular tonus at the time of swallowing is high, the initial decline is of long duration and constitutes the entire complex (Figures 2A and 8A).

A comparison of the slopes of contraction and subsequent relaxation in the vestibule following a swallow showed that the rate of relaxation was 5 to 10 times as fast as that of contraction, a pressure drop of 20 cm. water often occurring in one second. No relationship between the two slopes in any given complex is seen when the two slopes are plotted against each other and the gradient sum test applied.

Moreover, the rates of fall in pressure in the vestibule which followed periods of contraction varied among individuals and were different at various times in the same normal subject (Figure 8B). The rapid decline in pressure may be due not to sudden relaxation of the vestibular musculature, but to the rapidity of equilibration of pressures in the distal portion of vestibule and the stomach at the time the lumina of these organs are in continuity.

DISCUSSION

These studies confirm the previous work of others that there are two separate functional segments of the normal human esophagus: the body (upper eight-ninths) and the vestibule (lower one-ninth) (5, 6, 8, 10). Also, our observations and interpretations of the motility characteristics of the body associated with deglutition are for the most part in agreement with those of others (4, 5). While changes in vestibular pressure, particularly with swallowing, have been shown previously, our measurements demonstrate greater fluctuation of pressure in this segment, particularly during rest, than previously noted. Fluctuations in resting pressure do not appear to be caused by shifting of the recording tip out of the vestibule, because such pressure changes may be seen to occur simultaneously when recording with closely placed tips in this segment. Similarly when recording within 1 cm. above or below this segment, the baseline continues to show the resting pressure of the esophageal body or stomach while the vestibular tracing shows these changes. Further clarification of such problems will require simultaneous catheterization and cineradiographic investigations. Another difference has been our finding that the motility of the vestibule during deglutition is in part dependent upon its pressure relative to that in the stomach or lower esophagus at the time of the swallow. This gradient, readily noted in the same recording channel by switching from vestibule to these adjacent segments, varied greatly, but pressure was al-

ways equal or higher in the vestibule. This characteristic fluctuation in resting pressure and its influence upon pressure changes during deglutition certainly are consistent with sphincteric activity.

Fyke, Code and Schlegel, using a single transducer, demonstrated slightly higher resting pressure in the vestibular segment than in the stomach. With composite tracings recorded by withdrawing this catheter, and also from recordings made with open-tipped catheters in tandem, these authors demonstrated not only that relaxation occurred in this area with deglutition, but also that there was a rise in pressure above baseline for about 13 seconds thereafter. Analysis of their tandem record shows a fluctuation of 40 cm. H_2O in the vestibule during deglutition. They concluded that this behavior warranted designation of this segment as the "esophago-gastric sphincter" (10). Creamer, Fyke, Code and Olsen have reported similar findings with an electromagnetic transducer or an open-tipped catheter connected to a manometer (9). As further evidence, Fyke and co-workers cited the fact that pressure in this area relative to fundic pressure became greater in the head-down position (10).

Sanchez and associates, using open-tipped, water-filled catheter and electromanometers, have reported that the vestibule demonstrated motor behavior different from the body of the organ. Later, Ingelfinger mentioned "receptive relaxation of the vestibule with swallowing" (14). More recently, Fleshler, Hendrix, Kramer and Ingelfinger working in the same laboratory, have reported that there is an elevated resting pressure which disappears with or immediately after onset of body peristalsis (15). In addition, this segment resisted hydrostatic force. From these findings the authors concluded that the vestibule behaves as a sphincter. Our studies also indicate that the vestibule operates as a sphincter capable of rapid, vigorous contraction and relaxation and warrants designation as the "esophago-gastric sphincter."

Rapid changes in pressure in any short segment of gut pose two problems in recording, particularly when the walls are in apposition to each other and the lumen is completely obliterated. One is maintenance of the recording tip in the area for prolonged periods, and the other is the reliability of the recording, even if one is certain that the recording tip has not been dislodged. The first problem is largely solved by the catheter system employed in these studies which permits rapid localization of the sphincter without moving the catheter.

Since the vestibule may often be only 2 cm. in length, the generation of high pressures will tend to displace any catheter whose terminus is in the area, especially if there is a bulky tip at its end. As previously noted, bulkiness of catheters affects accuracy of pressure recording. It is also probable that a sudden increase in tone will dislodge any contents of the lumen, including a catheter tip, into the body of the esophagus above or into the stomach below. Frequent movement of the catheters by the observer to assure proper positioning may also cause retching or coughing which may displace the tips.

These difficulties are largely obviated by the six-tube system with closely approximated lateral openings, which makes possible a set of catheters of uniform diameter free of bulky recording tips, the terminus of the system residing in the stomach. Discomfort and retching are thus minimized. In addition, by means of switching the stopcock settings between the closely approximated lateral openings, small shifts in position can be adjusted for, and continuous recording for several hours is possible without moving the tubes. In this way one is better able to characterize and record continuously the motor behavior of the distal 2 to 5 cm. of the esophagus.

The sustained obliteration of the lumen which occurs at a sphincter interferes with reliable recording and is not easily obviated by changing the catheter design. Even assuming that one is recording constantly from the sphincteric region, the deficiencies of the measuring system have to be taken into account. In other hollow viscera the open-tipped catheters attached to the electromanometers always reside in a fluid-filled loop. Changes in pressure exerted on the fluid in the lumen are, therefore, transmitted through the catheters. In a sphincter, however, if all the gas or liquid is squeezed out when the lumen is obliterated, there is no medium on which pressure can be exerted. Instead, the opening of the catheter becomes

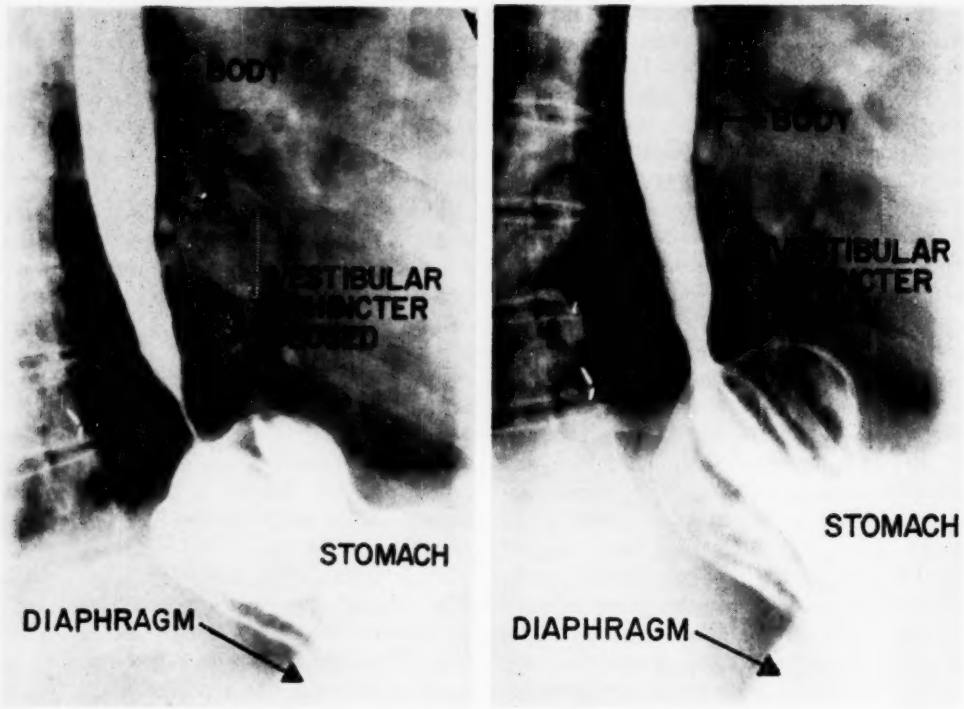

FIG. 9. A BARIUM FILLED SLIDING HIATUS HERNIA, ILLUSTRATING THAT THE SPHINCTERIC ACTION IS INDEPENDENT OF THE DIAPHRAGM

plugged at its distal end as the mucosa of the sphincter occludes it, or even protrudes into it.

The electromanometer is designed to register pressure changes induced at the end of a catheter to which it is attached by means of small volume displacements of the liquid in the system. Once the opening of a catheter becomes completely occluded by a "membrane" such as the mucosa of the obliterating wall, there may be failure of the electromanometer to register the exact height of the tonus change in the musculature, since there will be no displacement of liquid after the time of complete occlusion of the tip. However, if the system employs a constant infusion pump, a rise in pressure will be recorded since fluid is entering at the proximal end without any distal escape. This rise can only reach the level of that exerted at the distal opening by the sphincteric musculature. If it exceeds this level, it would force the mucosa away from the orifice, causing fluid to escape, and the pressure in the catheter could go no higher. Thus, the pump makes possible a much closer approximation to the actual pressure being exerted by a segment contracting strongly enough to produce occlusion. Furthermore, even when the tip is not occluded, the pump tends to keep the system continuously free of the cushioning effect of air bubbles.

The ability to record pressures from this short segment over long periods makes it possible to study the area in greater detail and to observe the coordinate action of the proximal and distal esophagus during the resting state, swallowing and drug administration. It is also possible to differentiate the sphincter pattern from the effect of the diaphragm by recording above, at and below the diaphragm simultaneously.

Aside from its pattern of contraction and relaxation during deglutition, there is other evidence that the vestibule constitutes an intrinsic sphincter. A short, contractile segment at the junction of the esophagus and the stomach may be observed

fluoroscopically and has been clearly demonstrated by Wolf and co-workers with cinemaradiography (16). Figure 9 shows respresentative roentgenograms to illustrate that the action of this segment is independent of the diaphragm. In the case illustrated, pressure studies of the vestibule showed a normal resting tone and a coordinated response to deglutition.

This physiologic sphincter cannot be readily identified morphologically as a localized muscular ring. In his careful study, Lerche (17) succeeded in finding a small band of muscle, but this was microscopic in size. The ease of passage of a bolus through this segment is attributable to reflex relaxation and this behavior is more consistent with the behavior of an intrinsic sphincter than of a valve. The relationship of the sling action of the right crus of the diaphragm (18) to the function of the intrinsic sphincter has not been evaluated by the present method. However, this sling action is probably unnecessary for, and unrelated to, normal sphincteric activity. Under physiological conditions the respirations occur more frequently than do the openings and closings of the sphincter. In addition, breath holding has little influence on the measurements of sphincteric activity, which also continues in the presence of hiatus hernia (Figure 9).

SUMMARY

1. A technique of esophageal catheterization is described for measuring the motility of the esophagus. The method employs electromanometers, a constant infusion pump, and a specially prepared set of six catheters connected to three 3-way stopcocks. The advantages of this system for pressure measurements from a sphincter are detailed.

2. The pattern of pressure changes obtained from the vestibule is different from that of the body of the esophagus both at rest and during deglutition, indicating that each of these is a separate functioning segment.

3. At rest, there are changing tonus levels in the vestibule, often with sudden generation or release of high pressures. These patterns are independent of those seen in the body of the esophagus and influence the type of response obtained in this segment during swallowing.

4. At the onset of swallowing, the vestibule briefly relaxes and, as peristalsis reaches this segment, a positive pressure wave is developed, following which relaxation again occurs. However, if vestibular resting pressure closely approximates that in the stomach, this initial relaxation does not occur. Conversely, when resting pressure is high, the drop in pressure is greater and so prolonged as to comprise the entire vestibular complex.

5. These motor patterns at rest and during swallowing indicate that the vestibule behaves as an intrinsic sphincter and should be referred to as the esophago-gastric sphincter.

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THE METABOLIC FATE OF C^{14} LABELED PENTOSES IN MAN

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The role of pentoses in intermediary metabolism has received much attention within the past few years. The phosphate esters of D-ribose, D-ribulose and D-xylulose have been identified as key intermediates in the pentose phosphate pathway of glucose metabolism in both plant and mammalian tissue (1). More recently, another alternate pathway of glucose dissimilation, the uronic acid pathway, has been described in which L-xylulose, xylitol and D-xylulose are intermediates (2).

Several pentoses have been identified in normal urine: the aldopentoses xylose, arabinose and ribose (3, 4) and the ketopentoses D-ribulose and L-xylulose (5). Large quantities of the latter sugar are excreted in the disease essential pentosuria (6, 7), which appears to be due to a defect in the further conversion of L-xylulose to the sugar alcohol xylitol (8). In addition to L-xylulose Touster and Harwell (9) have also reported the isolation of L-arabitol from the urine of pentosuric subjects.

Because of increasing awareness of the biological importance of the pentoses, certain aspects of pentose metabolism in man have been investigated in this laboratory. Previous reports have described the physiological disposition of large quantities of infused pentoses (10), the effect of insulin on blood levels of infused pentoses (11) as well as studies of the metabolism of D-ribose (12). This paper reports some observations on the fate of C^{14} labeled D-xylose, D-lyxose, D-arabinose and L-arabinose administered in trace quantities intravenously to normal human subjects. Some of the previously published data (12) on C^{14} ribose metabolism are included for purposes of comparison.

METHODS

Five experiments were performed in four normal male volunteers aged 18 to 21. Each subject had been on a 250 Gm. carbohydrate diet for at least three days and was fasted overnight prior to study.

Five μc. of each sugar dissolved in 250 ml. of normal saline were infused over a 15 minute period. Expired air samples were collected for 4 minutes at 15 to 60 minute intervals for 6 hours. Urine samples were collected at hourly intervals for 6 hours and pooled thereafter till 24 hours had elapsed from the start of the experiment. A urine sample was obtained at the twenty-fifth hour to determine if C^{14} was still being excreted. Urine was preserved by freezing at $-20°$ C. Blood was collected at 20 minute intervals.

Methods for the collection and C^{14} counting of expired CO_2, for preparation and radioactive assay of blood samples, and C^{14} counting and chromatography of labeled compounds in urine have previously been described (12).

The C^{14} pentoses D-xylose, D-lyxose, D-arabinose, L-arabinose and D-ribose (Figure 1) with specific activity of 0.67, 1.65, 1.65, 1.11, 2.6 and 2.01 μc. per mg., respec-

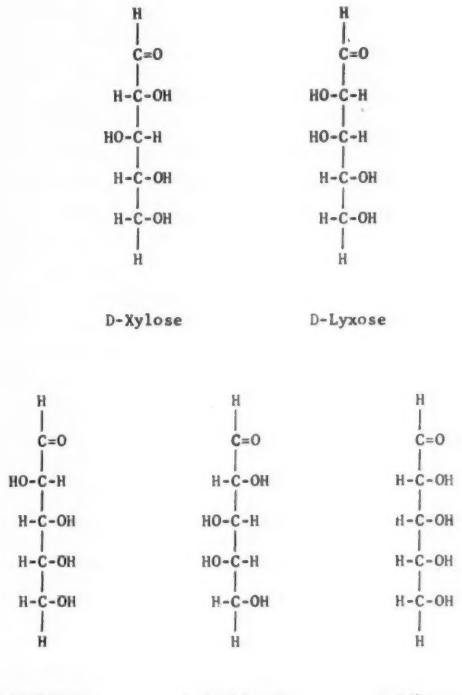

FIG. 1. STRUCTURAL FORMULAE OF PENTOSES STUDIED

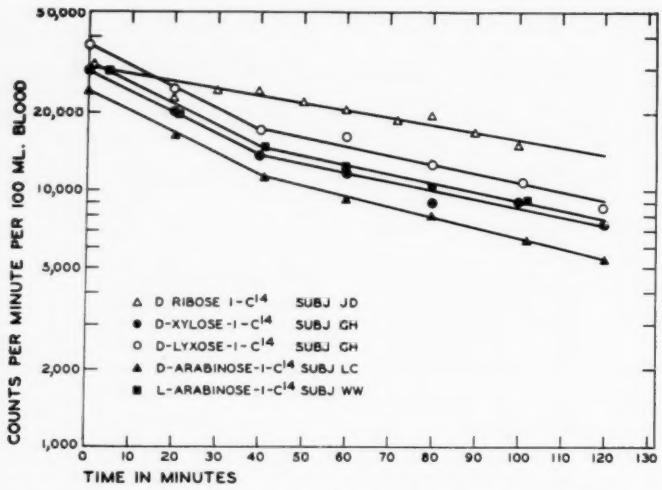

FIG. 2. C^{14} DISAPPEARANCE FROM BLOOD AFTER 5 μC. INTRAVENOUSLY

tively, were all labeled in the first carbon. They were obtained from Dr. H. Isbell of the National Bureau of Standards. The authenticity of these sugars was established by chromatographic comparison with known pure unlabeled pentoses.

RESULTS

The disappearance of C^{14} from blood

The C^{14} blood levels measured following infusion of the various pentoses are plotted on semilogarithmic coordinates in Figure 2. The straight lines obtained indicate that the removal of C^{14} occurs according to first order kinetics. During the first 30 minutes, there appears to be a deviation from the linear logarithmic curve finally established, a finding which probably represents a temporary distribution gradient between blood and tissues. One cannot exclude the possibility, however, that the initial curve represents pentose disappearance while that established after 30 minutes represents the composite disappearance curve of C^{14} pentose and C^{14} metabolites which have entered the blood. After infusion of large quantities of pentose, the disappearance curve of pentose from blood constructed from chemical analysis reveals a similar deviation during the first 30 minutes from the final linear curve (10).

A rate constant for disappearance of C^{14} from blood may be calculated from a knowledge of the biological half-time. In Table I are given the proportional rate constants expressed as K^1, the per cent C^{14} disappearing per minute for the linear curves established after 30 minutes. The rates of removal have a narrow range of 0.68 to 0.92 per cent per minute.

TABLE I

Disposition of C^{14} after infusion of labeled pentose

Subject	Sugar	% Dose expired as $C^{14}O_2$ in 6 hrs.	% Retained* C^{14} expired as $C^{14}O_2$ in 6 hrs.	Urinary C^{14} in 24 hrs.	Disappearance of C^{14} from blood
				% dose	*%/min. (K^1)*
G. H.	D-xylose	16	23	35	0.77
G. H.	D-lyxose	14	38	72	0.81
L. C.	D-arabinose	19	38	57	0.92
W. W.	L-arabinose	0.8	4	85	0.77
J. D.	D-ribose	48	50	10†	0.68

* Retained activity equals dose minus six hour urinary excretion.
† Five hour excretion; no activity detected after this time by methods employed.

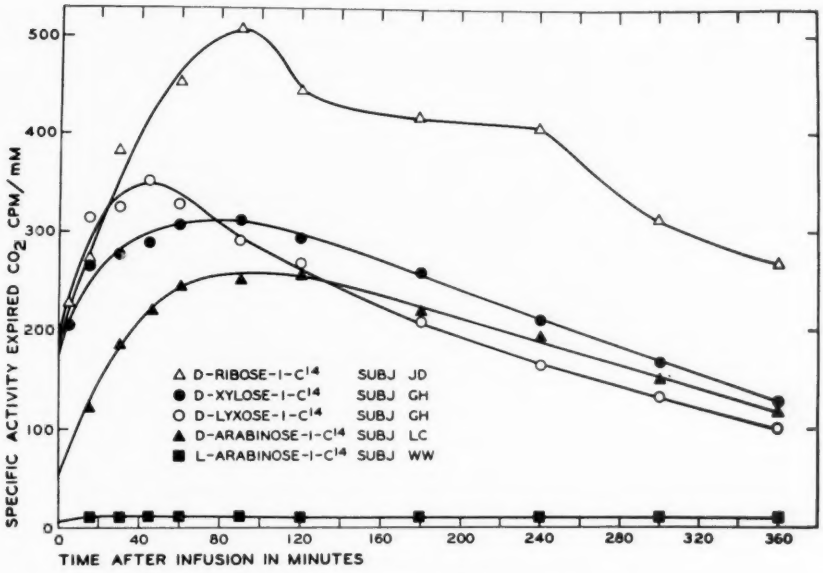

FIG. 3. EXPIRED $C^{14}O_2$ AFTER 5 μC. C^{14} PENTOSE INTRAVENOUSLY

The rate constants for the disappearance from blood of the C^{14} of D-xylose, D-lyxose and D- and L-arabinose are very similar to those calculated from the curves of disappearance of pentose determined chemically, following infusion of large quantities of sugar. These ranged from 0.77 to 1.58 per cent per minute (10). However, ribose chemically determined has been shown to be cleared from blood with rate constants much larger than the 0.68 recorded for C^{14}. For example, 3 Gm. of infused ribose was removed from blood at 11 per cent per minute (12).

Expired $C^{14}O_2$ after administration of C^{14}pentose

The specific activity of expired $C^{14}O_2$ is shown in Figure 3. Very little radioactivity was found in CO_2 following L-arabinose injection though significant amounts of $C^{14}O_2$ were derived from the first carbon of the other sugars. Quantitative data for C^{14} excretion via CO_2 are shown in Table I.

In six hours only 0.8 per cent of the dose of C^{14} L-arabinose appeared via this route whereas 48 per cent of the injected ribose radioactivity could be found. The $C^{14}O_2$ derived from D-xylose, D-lyxose and D-arabinose ranged from 14 to 19 per cent of the dose. Since much C^{14} appears in urine perhaps a more accurate appraisal of $C^{14}O_2$ derived from the pentose can be had if one calculates the per cent of retained C^{14} (the dose corrected for the six hour urinary excretion of C^{14}) in expired air. This is shown in Table I. It may be observed that a considerable portion of the first carbon of D-lyxose, D-xylose and D-arabinose actually present in the body is converted to CO_2.

Certain observations shown in Figure 3 are worthy of note. D-xylose-1-C^{14} and D-lyxose-1-C^{14} were administered to the same subject. Comparing the $C^{14}O_2$ excretion curves one finds that the dissimilation of D-lyxose with elimination of part of carbon one as $C^{14}O_2$ takes place at a faster rate and reaches an earlier peak than D-xylose. These two sugars differ only by the stereoconfiguration of carbon two (Figure 1). It is also apparent that D-ribose C^{14} differs from the other pentoses in the extent of oxidation of carbon one to CO_2 and the seemingly different mechanism is reflected in the shape of the ribose curve. Interpretation of the latter has received comment previously (12). The more extensive metabolism of ribose C^{14} as compared to the other pentoses may be related to its extensive conversion to D-glucose.

Urinary excretion of C^{14}

The per cent of the C^{14} dose appearing in urine for the 24 hour period after infusion is tabulated in Table I and shown graphically in Figure 4. The urinary excretion of C^{14} ranged from a total of 10 per cent for ribose to 85 per cent for L-arabinose. The low urinary excretion of ribose C^{14} appears to reflect the large amount of conversion to CO_2, the high renal excretion of C^{14} from L-arabinose reflecting the minute conversion to CO_2. Although the dissimilation of the other pentoses D-xylose, D-lyxose and D-arabinose occurred with a similar quantity of $C^{14}O_2$ production there are marked differences in the urinary excretion of C^{14} (Table I).

The cumulative urinary excretion curves of C^{14} indicate a similarly rapid initial rate of egress of C^{14} derived from D-lyxose and D- and L-arabinose. The early excretion rate of C^{14} from xylose and ribose is much slower. The C^{14} excretion from ribose reached a plateau after a few hours and that from xylose within 24 hours. This seems not to be the case for the other sugars for their curves suggest a continued C^{14} excretion beyond 25 hours.

In an attempt to determine whether the urinary C^{14} activity represented unaltered sugar, urine was chromatographed on paper and the C^{14} residing in bands corresponding to the sugar was eluted and counted. The results are shown in Table II. In all cases except for ribose the C^{14} in urine is only in part accounted for by unmetabolized sugar in the two hour postinfusion period studied. The quantity excreted as pentose appeared to decrease with time. Most striking is the finding that only 7 per cent of the C^{14} in the second hour urine of the subject receiving D-arabinose could be accounted for as the original sugar, whereas 64 per cent could be so detected during the first hour. These findings suggest that C^{14} containing material other than the injected sugar is present in the urine. The nature of this metabolite or metabolities remains to be identified.

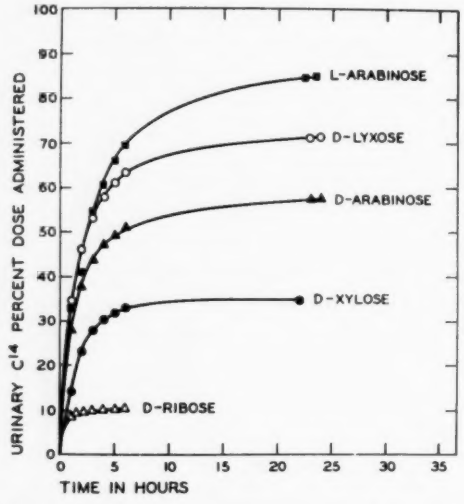

FIG. 4. URINARY C^{14} EXCRETION AFTER C^{14} PENTOSE ADMINISTRATION

TABLE II

Paper chromatography of C^{14} in urine of subjects receiving C^{14} pentose

Sugar	Solvent	% C^{14} in urine found in pentose area: 1st hour	% C^{14} in urine found in pentose area: 2nd hour
D-xylose	MEK HAC H_2O*	60	61†
	BuOH HAC H_2O‡		59
D-lyxose	MEK HAC H_2O	70	51
D-arabinose	MEK HAC H_2O	64	7
	BuOH HAC H_2O		7§
L-arabinose	MEK HAC H_2O	51‖	25
D-ribose	MEK HAC H_2O	92	

* Methylethyl ketone, acetic acid, water 6:1:1.
† Recovery of the infused C^{14} pentose co-chromatographed with this urine was 94 per cent.
‡ Butanol, acetic acid, water 4:1:5.
§ Recovery of the infused C^{14} pentose co-chromatographed with this urine was 102 per cent.
‖ Recovery of the infused C^{14} pentose co-chromatographed with this urine was 114 per cent.

DISCUSSION

Previous reports from this laboratory (10, 12) described the disappearance from blood and the urinary excretion of large quantities of D-xylose, D-lyxose, D- and L-arabinose and D-ribose given intravenously. The disposition of the C^{14} from the radioactive forms of these sugars given in tracer quantities permits some comparisons. In the present studies the disappearance of C^{14} from blood was observed to obey first order kinetics. The rate constants for all but ribose are very similar to those obtained by determination of orcinol reacting substance during the load experiments. As has been pointed out (12) the curve for the removal of ribose from blood constructed

from the orcinol assay differs considerably from that based on C^{14} counting data, a finding which appears to be explained by the rapid conversion of the C^{14} labeled carbon of ribose to blood glucose and other C^{14} labeled material (12).

Chromatographic studies suggesting that C^{14} labeled material other than pentose is present in urine indicates that the C^{14} disappearance curve from blood may represent not only pentose but may be a composite curve for disappearance of other C^{14} labeled substances derived from pentose. Since the orcinol reaction is not specific for pentoses, the curves determined after the load infusions (10) may also in part represent metabolites of the infused pentose. It may be, however, that the C^{14} in blood represents only a small quantity of nonpentose material but that the renal clearance of this unknown substance is greater than that of the pentose itself or that the nonpentose C^{14} is formed in the kidney and excreted as such. Under such circumstances the C^{14} disappearance from blood would be an accurate picture of the removal of pentose.

Certain differences between the urinary excretion of pentose as determined chemically after infusion of 5 to 20 Gm. amounts and the C^{14} excretion after the tracer dose of radioactive sugar can be pointed out. In the former experiments, the urinary excretion of xylose, lyxose and both arabinoses ranged from 27 to 60 per cent of the dose and averaged 44 per cent, no significant difference being observed between these pentoses (10). Ribose measured chemically in urine revealed an excretion of about 20 per cent of the dose but this figure depended on the amount administered. In the present studies 10 per cent of the ribose dose was excreted in the urine. The C^{14} excretion of the remaining pentoses varied with each sugar (Figure 4) and the per cent excreted via this route was over 60 per cent for D-lyxose and L-arabinose, being nearly quantitative for the latter pentose. Another dissimilarity between the results obtained chemically and C^{14} analyzed experiments may be observed. When 20 Gm. of D-xylose and D-lyxose were given to the same individual the urinary excretion of orcinol reactive substance was essentially the same for both sugars (10). When this comparison was made with C^{14} sugar there was a marked difference in C^{14} urinary excretion (Subject G. H.).

Chromatography of the urine in the studies reported here has revealed evidence for radioactive material other than the infused pentose. If one corrects the urinary C^{14} excretion for the actual pentose present in the first hour and the remainder of the C^{14} for the actual pentose determined in the second hour, it may be estimated that only 21 per cent of the dose of D-xylose, 20 per cent of D-arabinose, 42 per cent of D-lyxose and 33 per cent of L-arabinose were excreted as unaltered C^{14} labeled pentose.

The dissimilation of D-xylose, D-lyxose, D-arabinose and D-ribose in man has been found to occur with elimination of a fraction of carbon one as $C^{14}O_2$. Since these sugars have been given intravenously with almost immediate production of $C^{14}O_2$ it is highly unlikely that this metabolism is a result of intestinal bacterial activity. The catabolism of L-arabinose results in negligible amounts of $C^{14}O_2$. This observation based on an experiment in one individual raises the possibility that Subject W. W. given the L-arabinose-1-C^{14} did not metabolize L-arabinose to CO_2 because of an enzymatic deficiency which is not present in other humans. This seems unlikely in view of his ability to handle 20 Gm. infusions of L-arabinose in a manner similar to other normal subjects.

Few studies of catabolism of C^{14} labeled pentoses to $C^{14}O_2$ in other animals have been reported. Bloom (13) has shown $C^{14}O_2$ production from ribose in the rat. Bloom and Stetten, however, found that D-arabinose-1-C^{14} and D-arabinose-5-C^{14} did not give rise to significant yields of $C^{14}O_2$ when incubated with rat liver slices or when administered to intact rats or rabbits (14). Since D-arabinose-1-C^{14} gives rise to significant amounts of $C^{14}O_2$ in man it appears that man is different from these animals in this regard.

The total 24 hour recovery of the adminstered C^{14} may be estimated from the urinary recovery data, the amount of expired $C^{14}O_2$ and calculation of label in the bicarbonate pool at the end of six hours (15). This latter figure is about 5 per cent of the dose for D-lyxose, D-xylose and D-arabinose, 10 per cent for ribose and zero for L-arabinose. C^{14} of lyxose was almost quantitatively accounted for at about 90 per cent of the dose. Eighty-five per cent of the L-arabinose label was excreted solely in urine. From the C^{14} excretion curve for L-arabinose it appears that further label

was excreted after 24 hours. Eighty per cent of D-arabinose C^{14} and 68 per cent of ribose could be accounted for. This is a low estimate for ribose since there was continued $C^{14}O_2$ excretion beyond six hours and since other studies (12, 16) reveal a significant portion of the ribose C^{14} to be in the glucose pool. Only about 55 per cent of xylose C^{14} could be accounted for. The fate of the remaining label is unknown.

Whether the pentoses are in some part converted to glucose is a question of general interest. After infusion of D-xylose and L-arabinose in man a rise in blood glucose has been observed (10). Little or no change occurs upon infusion of D-arabinose or D-lyxose, whereas D-ribose has been observed to cause marked decreases in blood glucose level (17). Despite the hypoglycemia induced by ribose, D-ribose-1-C^{14} has been shown to be converted to blood (16) and urinary glucose (12) via the pentose phosphate pathway. From the results reported here it appears that though the blood glucose level may rise after L-arabinose infusion, this is not due to conversion to glucose for nearly all of the label is renally excreted and none appears as $C^{14}O_2$. No direct evidence is at hand to state whether conversion to glucose is a fate of D-xylose, D-arabinose or D-lyxose in man. Comparing the $C^{14}O_2$ excretion of these sugars with that of D-ribose which is known to be glucogenic one might suspect that these sugars are converted to glucose minimally or not at all. In the intact mouse Hiatt (18) has observed slight conversion of xylose-1-C^{14} to glycogen via the pentose phosphate scheme but virtually no conversion of D- or L-arabinose.

It is possible that D-xylose, D-arabinose and D-lyxose might enter the pentose phosphate pathway. In bacteria, for example, a xylose isomerase has been isolated (19) which converts this sugar to D-xylulose which may be phosphorylated to xylulose 5-phosphate (20). It has also been demonstrated in microorganisms that D-lyxose may be isomerized to D-xylulose (21). From the $C^{14}O_2$ excretion curves presented, if these sugars were converted to D-xylulose and thence entered the pentose phosphate pathway the early peak labeling of $C^{14}O_2$ shown for D-lyxose would indicate that the lyxose isomerase enzyme was more efficient than the xylose isomerase. In *E. coli* D-arabinose has been shown to form D-ribulose (22), which after phosphorphorylation could enter the path to glucose formation.

Pathways to $C^{14}O_2$ from pentose other than via conversion to glucose exist for pentoses in bacteria. D-Xylulose may undergo phosphorolysis to acetyl phosphate and triose phosphate (23). D-Arabinose may be catabolized by the following scheme (24): D-arabinose→D-arabonic acid→2 keto, 3-deoxyarabonic acid→pyruvic acid plus glycolic acid.

From our data L-arabinose appears to be catabolized but to products other than CO_2. In bacteria, the pathway L-arabinose→L-arabinolactone →L-arabonic acid→α ketoglutarate has been described (25). Since α ketoglutarate gives rise to CO_2, if this pathway were operative in man the end product would be one prior to the keto acid. Apparently, the sequence L-arabinose→L-ribulose →D-xylulose-5-phosphate reported in bacteria (26, 27) is absent in man for this would give rise to $C^{14}O_2$ via the pentose phosphate pathway. Another alternative for the fate of L-arabinose may be conversion to L-arabitol, a substance which has been isolated from the urine of pentosuric individuals (9).

SUMMARY

Carbon 14 labeled pentoses have been infused intravenously to normal human subjects. The disposition of the label has been followed in blood, urine and expired air. It has been found that D-xylose, D-lyxose, D-arabinose and D-ribose are catabolized to some extent to $C^{14}O_2$. Much of the label appears in the urine in a form that is not the infused sugar. L-Arabinose gives rise to negligible amounts of $C^{14}O_2$. The possible pathways of metabolism of these sugars are described.

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THE EXCRETION OF *l*-MALIC ACID IN RELATION TO THE TRICARBOXYLIC ACID CYCLE IN THE KIDNEY*

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The tricarboxylic acid cycle, described by Krebs and Johnson (1), has become generally accepted as the final common pathway for the aerobic oxidation of carbohydrate, fat and protein in most tissues of a large number of animal species. For reference during the discussion that follows, this cycle is presented in its usual schematic form in Figure 1.

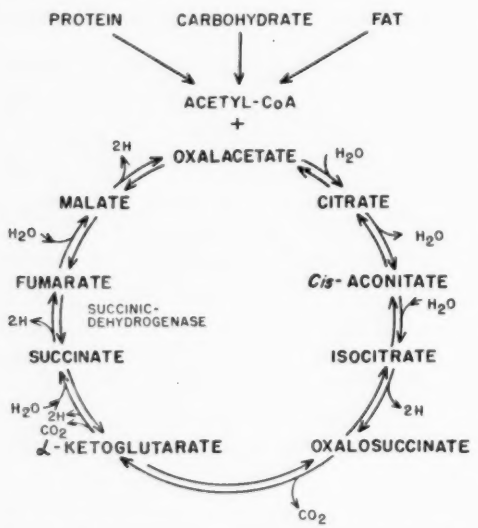

FIG. 1. DIAGRAMMATIC REPRESENTATION OF THE TRICARBOXYLIC ACID CYCLE

This sequence of biological oxidations is present in mammalian kidney, and all of its intermediate organic acid substrates have been identified at one time or another in urine of lower animals and man (2). Under certain circumstances the excretion of some of these acids shows remarkable variations. For instance, in metabolic alkalosis the rate of excretion of citrate and to a lesser extent of α-ketoglutarate is greatly elevated (3–6). On the other hand, in metabolic acidosis, or more precisely in conditions favoring intracellular acidosis such as potassium deficiency (4, 5) or the administration of Diamox® (7), the rate of excretion of these same acids is reduced almost to zero.

The way in which fluctuating acid-base balance causes these changes in organic acid excretion is not certain. However, a large body of indirect evidence supports the concept that some of these acids, besides undergoing glomerular filtration and tubular reabsorption, are produced in the renal tubular cells and secreted into the urine. It is supposed that it is this tubular synthesis that is sensitive to pH changes within the cells and thus affects the changing rates of organic acid excretion. Such would appear to be the case for citrate and α-ketoglutarate although direct evidence for their tubular secretion is not available. In the case of malic acid, however, such evidence is available and constitutes the main subject of the present paper.

In 1953 Craig, Miller, Owens and Woodward reported (8) that during the intravenous infusion of sodium salts of either α-ketoglutaric or succinic acids in dogs there was both glomerular filtration and net tubular secretion of malic acid. Under these circumstances they found that the infusion of malonic acid, the succinoxidase inhibitor, caused an abrupt change in the pattern of malic acid excretion to one of glomerular filtration with net tubular reabsorption.

It is the purpose of the present paper to report experiments on malic acid excretion in the dog that confirm and extend these findings of Craig and co-workers and include a description of the varying patterns of malic acid excretion during

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infusion of a number of the other cycle acids including fumarate and *l*-malate itself. These results will then be discussed in relation to the general problem of organic acid excretion and the biochemical function of the tricarboxylic acid cycle in the kidney.

METHODS

The experiments were performed on unanesthetized, hydrated female mongrel dogs trained to lie on an animal table while loosely restrained. Sodium salts of the acids of the tricarboxylic acid cycle were administered by constant intravenous infusion. Standard clearance techniques were used to quantify the rates of glomerular filtration and urinary excretion of the several acids studied. The creatinine clearance was used as a measure of glomerular filtration rate by methods described previously (9). Intravenous infusions were administered at a constant rate of 3 ml. per minute. Malic acid was determined essentially by the method of Hummel (10) which gave excellent quantitative malate recoveries from both plasma and urine, even in the presence of considerable amounts of each of the other cycle substrates. Succinate in tungstic acid filtrates of plasma and in diluted urines was determined enzymatically after ether extraction according to the method described by Umbreit, Burris and Stauffer (11). Citrate was measured with slight modification according to Natelson, Pincus and Lugovoy (12). Malate was found to be completely filtrable from plasma over a wide range of concentrations. The ultrafiltration studies were carried out first with Visking cellophane bags in air. The results of these studies were compared and found to agree with those from dialysis against phosphate buffer as described by Taggart (13).

TABLE I

Experiment illustrating pattern of l-malic acid excretion and effect of malonate during infusion of sodium citrate in dog

Total concurrent time	Glomerular filtration rate	*l*-Malic acid				
		Plasma	Filtered	Excreted	Secreted	Reabsorbed
min.	*ml./min.*	*μg./ml.*	*μg./min.*	*μg./min.*	*μg./min.*	*μg./min.*
	Prime—Sodium citrate, 0.10 Gm./Kg. + creatinine, 0.15 Gm./Kg. *Infuse*—Sodium citrate, 1.80 mg./Kg./min. in 0.9% NaCl + creatinine, 1.8 mg./Kg./min.					
30–40	110.0	3.37	375	925	554	
40–50	113.4	3.37	383	900	517	
50–60	119.2	3.13	373	825	452	
	Prime—Disodium malonate, 0.41 Gm./Kg. *Infuse*—As before + malonate, 1.26 mg./Kg./min.					
90–100	114.6	3.63	416	288		128
100–110	101.4	3.50	355	250		105

TABLE II

Experiment illustrating pattern of l-malic acid excretion and effect of malonate during infusion of sodium α-ketoglutarate in dog

Total concurrent time	Glomerular filtration rate	*l*-Malic acid				
		Plasma	Filtered	Excreted	Secreted	Reabsorbed
min.	*ml./min.*	*μg./min.*	*mg./min.*	*mg./min.*	*mg./min.*	*mg./min.*
	Prime—α-ketoglutaric acid, 0.49 Gm./Kg. (neutralized with NaOH) + creatinine, 0.15 Gm./Kg. *Infuse*—α-ketoglutaric acid, 6.7 mg./Kg./min. (neutralized with NaOH) + creatinine, 1.9 mg./Kg./min. in 0.9% NaCl					
30–40	78.0	17.8	1.39	3.32	1.93	
40–50	82.5	18.6	1.53	3.23	1.70	
50–60	86.5	19.2	1.66	2.68	1.02	
	Prime—Disodium malonate, 0.41 Gm./Kg. *Infuse*—As before + malonate, 4.00 mg./Kg./min.					
90–100	75.0	16.5	1.24	0.92		0.32
100–110	78.0	15.1	1.18	0.86		0.32
110–120	72.8	13.8	1.01	0.68		0.33

RESULTS

The pattern of malate excretion during infusion of citrate, α-ketoglutarate, or succinate

Table I shows the details of an experiment illustrating the pattern of *l*-malate excretion during the infusion of sodium citrate. Because of its toxicity when infused at high rates, citrate administration had to be limited. Nevertheless it is seen that its infusion produced a small but definite elevation in plasma malate concentration to some 3.0 to 3.4 μg. per ml.; endogenous malate levels vary from 0.5 to 2 μg. per ml. Furthermore, in the first three periods malate excretion was some 500 μg. per minute greater than its filtration, indicating that a rather large tubular secretion of malate occurred during citrate infusion even in these relatively small amounts.

Effect of malonate. Since the secretion of malate here must depend first on its synthesis from citrate via the tricarboxylic acid cycle steps shown in Figure 1, it would be expected that malonic acid, the succinoxidase inhibitor, would block this part of the malate excretory process. Indeed, this is what happened. The high rate of tubular malate secretion seen in the first three periods disappeared after malonate and there appeared instead a net tubular reabsorption of malate amounting to 128 to 105 μg. per minute (last three periods). Thus there appears to be, as Craig and associates found, a bidirectional movement of malate across the renal tubule in the dog; the reabsorptive limb of the process becomes evident when the net tubular secretion is blocked after malonate (8).

Experiments similar in design to that shown in Table I were performed with α-ketoglutarate and succinate. We wished to see whether the pattern of malate excretion seen with citrate was also observed during infusion of these two substrates which would likewise have to utilize the succinoxidase system in order to give rise to malate. Results of experiments of this type are shown with α-ketoglutarate in Table II and succinate in Table III. It is apparent in both these experiments that the pattern of malate excretion during either α-ketoglutarate or succinate infusion is *qualitatively* similar to that seen when citrate was substrate. Plasma malate is elevated as, consequently, is its rate of glomerular filtration. There is a large net tubular secretion which in each case is changed to a net tubular reabsorption after the addition of malonate to the infusion.

With α-ketoglutarate and succinate there are, however, striking *quantitative* differences in malate excretion from that seen with citrate. For instance, with α-ketoglutarate and even more so with succinate, plasma malate levels as well as rates of tubular secretion and tubular reabsorption (after malonate) are considerably higher than with citrate. Whether the increasing size of these functions with the three substrates bears any relation to their progressive nearness to succinoxidase in the cycle is a possibility but not one sub-

TABLE III

Experiment illustrating pattern of l-malic acid excretion and effect of malonate during infusion of sodium succinate in dog

Total concurrent time	Glomerular filtration rate	*l*-Malic acid				
		Plasma	Filtered	Excreted	Secreted	Reabsorbed
min.	*ml./min.*	*μg./ml.*	*mg./min.*	*mg./min.*	*mg./min.*	*mg./min.*
	Prime—Succinic acid, 0.56 Gm./Kg. (neutralized with NaOH) + creatinine, 0.08 Gm./Kg. *Infuse*—Succinic acid, 5.1 mg./Kg./min. (neutralized with NaOH) + creatinine, 0.7 mg./Kg./min. in 0.9% NaCl					
30–40	101.5	33.6	3.41	6.61	3.20	
40–50	104.0	31.2	3.25	8.26	5.01	
50–60	99.0	39.0	3.86	8.26	4.40	
	Prime—Disodium malonate, 0.41 Gm./Kg. *Infuse*—As before + malonate, 3.8 mg./Kg./min.					
90–100	75.0	38.4	2.88	1.29		1.59
100–110	76.0	30.0	2.92	1.50		0.79
110–120	73.8	34.8	2.57	1.41		1.16

TABLE IV

Experiments on the same dog showing higher rate of malate reabsorption during fumarate than during l-malate infusion

Total concurrent time	Glomerular filtration rate	*l*-Malic acid			
		Plasma	Filtered	Excreted	Reabsorbed
min.	*ml./min.*	*mg./ml.*	*mg./min.*	*mg./min.*	*mg./min.*
No. 20 fumarate infusion					
Prime—Sodium fumarate, 0.19 Gm./Kg. + creatinine, 0.15 Gm./Kg. *Infuse*—Sodium fumarate, 1.8 mg./Kg./min. + creatinine, 1.8 mg./Kg./min. in 0.9% NaCl					
30–40	111.6	0.336	37.5	7.92	29.6
40–50	111.4	0.331	36.9	9.00	27.9
50–60	109.0	0.327	35.7	9.00	26.7
No. 21 malate infusion					
Prime—Sodium *l*-malate, 0.15 Gm./Kg. + creatinine, 0.15 Gm./Kg. *Infuse*—Sodium *l*-malate, 1.36 mg./Kg./min. + creatinine, 1.8 mg./Kg./min. in 0.9% NaCl					
30–40	93.0	0.420	39.0	35.0	4.0
40–50	93.4	0.390	36.5	30.4	6.1
50–60	98.3	0.336	33.0	27.1	5.9

ject to proof from these data. The relative smallness of the malate parameters during citrate infusion is most likely related to the fact that its cardiac toxicity limits the amount that can be given.

The experiments presented thus far are in complete agreement with the results of Craig and coworkers (8) and confirm their finding of a bidirectional malate movement in the tubule of the dog kidney, and extend that finding to include citrate as well as α-ketoglutarate and succinate as malate precursors in this phenomenon.

The pattern of malate excretion during infusion of fumarate or l-malate

It next became of interest to study the nature of malate excretion during infusion of substrates lying beyond the malonate-sensitive succinoxidase system in the cycle. Experiments were performed with two of these: fumarate and *l*-malate itself.

In the first experiment (No. 20) shown in Table IV sodium fumarate was given. This produced a marked elevation in plasma malate and, hence, in its glomerular filtration. In striking contrast to the earlier studies almost all the filtered malate was reabsorbed; no net tubular secretion was seen. In addition, the rate of tubular reabsorption of malate was much greater than that observed during malonate infusion with either citrate, α-ketoglutarate, or succinate.

In the second experiment (No. 21) shown in Table IV sodium *l*-malate was infused in sufficient amounts to achieve plasma levels and filtered loads analogous to those achieved in the fumarate infusion studies. One can see that with malate infusion, as with fumarate, there was likewise glomerular filtration and net tubular reabsorption of malate; no tubular secretion was seen. However, in striking contrast to the fumarate experiment, the reabsorptive rate of malate was much lower, being only 4 to 6 mg. per minute, compared to 27 to 29 mg. per minute at analogous malate loads during fumarate infusion.

Experiments like No. 21, Table II, were repeated and rates of malate filtration, excretion and reabsorption during *l*-malate infusion were measured over a wide range of plasma malate levels. The massed data of six such experiments are presented in Figure 2. Although the curves were drawn through the points by inspection, it is apparent that below filtered loads of 4 to 5 mg. per minute reabsorption of filtered malate is essentially complete. However, as plasma level rises a maximum reabsorptive rate (Tm malate) is reached and remains relatively constant over a wide range of filtered malate loads. Thus in exhibiting a "Tm" (during *l*-malate infusion), the

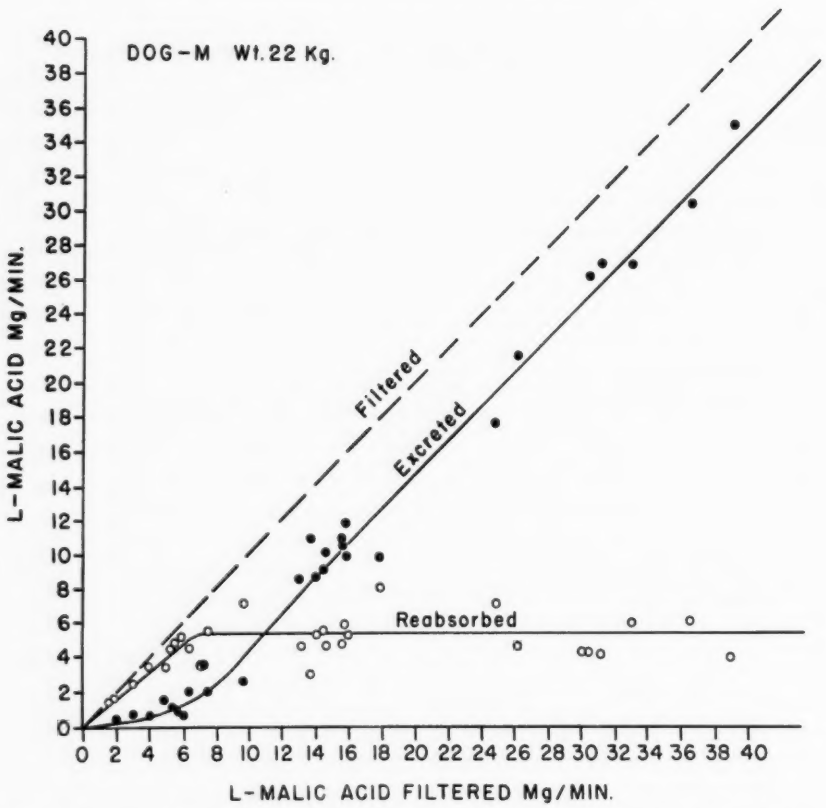
DOG-M Wt. 22 Kg.
L-MALIC ACID Mg/MIN.
L-MALIC ACID FILTERED Mg/MIN.
Filtered
Excreted
Reabsorbed
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40

FIG. 2. EXCRETION AND REABSORPTION OF MALATE IN RELATION TO FILTERED MALATE LOAD

malate reabsorptive mechanism resembles those of glucose, phosphate, sulfate and some amino acids.

Catalytic effect of fumarate on malate reabsorption

As was noted above in the experiments of Table IV, the rate of malate reabsorption during fumarate infusion is some five to six times higher than it is with equivalent malate loads during sodium *l*-malate infusion. In attempting to design further experiments to clarify the nature of this difference. we were reminded of the catalytic "sparking" effect of fumarate in isolated tissues oxidizing pyruvate (14, 15). We wondered whether fumarate could exert a similar catalytic effect on malate reabsorption by "sparking" the oxidative energy production in the renal tubular cells.

To test this hypothesis, experiments such as that presented in Figure 3 were performed. Sodium *l*-malate was infused at rates similar to those in Experiment No. 21, Table IV. Analogous filtered loads of malate were reached, and, as is seen in the first two bars in the bottom set, the malate reabsorptive rate was also similar at about 4 mg. per minute. Now while maintaining this malate infusion constant, sodium fumarate, in one-sixtieth the molar amount of infused malate, was added to the infusion and two more clearance periods were taken. With the fumarate addition the rate of malate reabsorption doubled or tripled as can be seen in the second two bars of the lower set. The massed data of two additional and similar experiments are presented in Figure 4. In each of these, two control periods were followed by four periods with fumarate addition. The black dots show malate reabsorption during control periods; these group around the dashed line representing Tm malate taken from Figure 2.

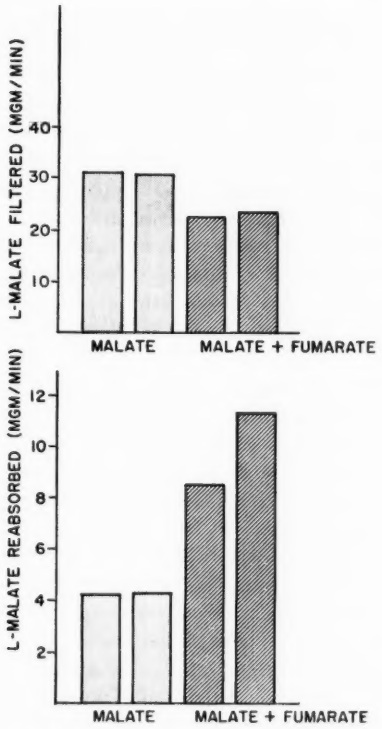

FIG. 3. AN EXPERIMENT ILLUSTRATING THE CATALYTIC EFFECT OF FUMARATE ON MALATE REABSORPTION

The open circles represent rates of malate reabsorption during fumarate addition. In all cases malate reabsorptive rates were significantly higher during periods when fumarate was infused along with the malate.

The word "catalytic" to describe this effect of fumarate is used advisedly because these quantities of fumarate, of themselves, would never lead to malate loads or reabsorptive rates such as these. In addition, the effect is the more striking because it occurs with an actual decline in the filtered malate load (Figure 3), the result of a general reduction of plasma malate seen during the infusion of small doses of fumarate with *l*-malate.

DISCUSSION

The experiments presented here support the theoretical conclusion that the tricarboxylic acid cycle is involved in the tubular synthesis and secretion of malic acid, as well as in its reabsorption

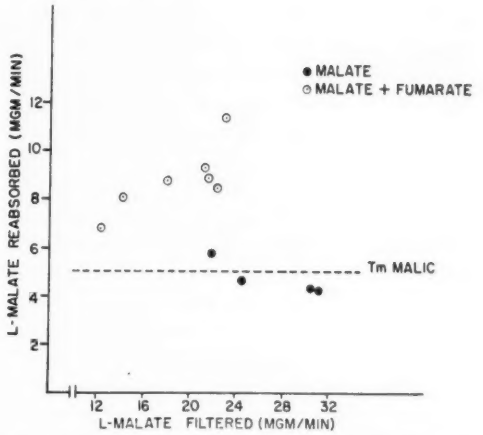

FIG. 4. MASSED DATA OF TWO EXPERIMENTS SHOWING EFFECT OF FUMARATE ON MALATE REABSORPTION

from the glomerular filtrate. These relations as visualized at present are summarized schematically in Figure 5. The upper tubular cell represents the mechanisms of malate reabsorption and the lower one, those of malate synthesis and secretion. Plasma malate level rises with infusion of citrate, α-ketoglutarate, succinate, fumarate, and, of course, *l*-malate itself. The malate formed from these several substrates in various tissues, or administered preformed, is freely filtrable and passes into the glo-

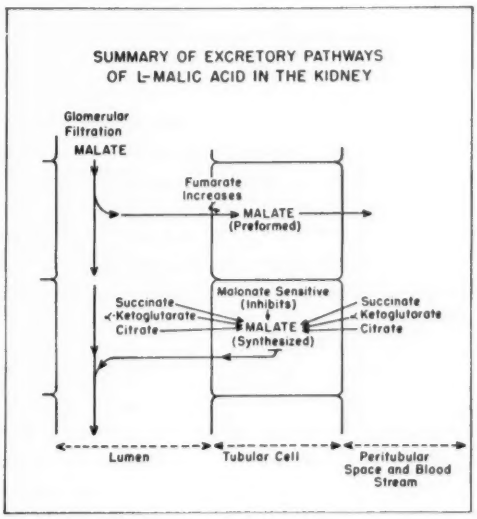

FIG. 5. SUMMARY OF THE PATHWAYS OF MALATE EXCRETION IN THE KIDNEY

merular filtrate where it undergoes tubular reabsorption (upper cell). This mechanism exhibits a Tm during infusion of *l*-malate. It is unaffected by malonate. Catalytic amounts of fumarate accelerate the reabsorptive capacity of the tubules for malate some two- to threefold, and during the infusion of fumarate alone, malate reabsorption is five or six times as high as it is at equivalent loads during malate infusion. Substrates of the cycle such as citrate, α-ketoglutarate and succinate get into the tubular cell (possibly from both sides as shown in the lower cell) and are synthesized there into malate by a malonate-sensitive mechanism presumably involving the succinoxidase system. That citrate and α-ketoglutarate can gain entrance to the tubule cell from the lumen is known from the fact that tubular reabsorption of each acid has been observed (16, 17). These observations on the several mechanisms of malic acid excretion in the kidney raise several important points about the nature of the phenomena themselves and their relation to the larger problem of organic acid excretion, its function in the overall economy of the body, and the role of the tricarboxylic acid cycle in renal tubular function.

In measuring "reabsorption" or "secretion" by the clearance technique, one simply measures *net* difference between rate of glomerular filtration and urinary excretion of a substance. This measurement does not reveal multidirectional fluxes that may comprise the "net" movement, nor does it reveal the fate of a substance reabsorbed or secreted by the tubules. Thus with a metabolite such as malic acid the measurement of its "reabsorption" does not tell us whether it has been moved intact without utilization from glomerular filtrate to peritubular blood or whether it has been partly or completely "utilized" by the tubule cells. Therefore, the catalytic effect of fumarate on malate reabsorption could represent either a facilitation of its actual transtubular movement, an acceleration of its tubular "utilization," or both. To differentiate these possibilities, studies will be required in which one measures simultaneously excretion of malate by clearance techniques and its utilization by renal arteriovenous sampling. Although such studies have not appeared for malate, Cohen (17) has reported them for other metabolites: lactate, acetoacetate, pyruvate and α-ketoglutarate. These studies have clearly shown that renal "utilization" and "reabsorption" of certain metabolites occur simultaneously and are independently affected by various experimental manipulations.

It seems, at first, strange that malate secretion is observed during infusion of citrate, α-ketoglutarate, or succinate but not during administration of fumarate or malate. One must presume that with the former three substrates malate is synthesized in the tubule cells by reactions involving succinoxidase and reaches sufficiently high concentration there (above that in plasma and glomerular filtrate) to pass along a concentration gradient into luminal urine. The equilibrium constants of both *fumarase* and *malic dehydrogenase* strongly favor such accumulation of malate within the cell. The equilibrium constant of the fumarase step is 4.0 to 4.5 at pH 7.3 and 25° C. (18) in favor of malate, and the constant of the diphosphopyridine nucleotide (DPN)-linked malic dehydrogenase step at pH 7.2 and 22° C. is 2.33×10^{-5} (19), again strongly in favor of malate.

When fumarate or malate is infused, however, plasma levels of malate are much higher. Thus perhaps under these circumstances concentration differences between cell and lumen are not so great, tubular secretion does not occur, and the reabsorptive limb of the bidirectional malate system becomes predominant. Whether this explanation of the observed facts is correct must await further study, but at the moment it seems a plausible formulation.

During infusion of citrate and α-ketoglutarate, glomerular filtration and net tubular reabsorption of these acids have been observed (16, 17). Preliminary studies in our laboratory with succinate have produced succinate to creatinine clearance ratios varying from 0.78 to 1.25, indicating that this acid is probably excreted predominantly by glomerular filtration when it is infused in the normal dog.

In contrast, however, the pattern of excretion of citrate and α-ketoglutarate is different when other intermediates of the tricarboxylic acid cycle are infused or when acid-base balance is altered. And, as with malate, the evidence, although indirect, supports the concept that they are synthesized from precursors in the tubule cells and secreted into the urine. For instance, in 1937 Orten and Smith (20) showed in dogs that the infusion of succinate, malate, malonate, or maleate

causes a remarkable increase in citrate excretion. A year later Krebs, Salvin and Johnson (21) reported similar studies with the rabbit and rat and explained Orten and Smith's results in terms of the "citric acid cycle" which by then had been clearly formulated in Kreb's laboratory. Krebs and coworkers (21) also explained Orten and Smith's malonate effect on the basis of its succinoxidase inhibition: citrate simply accumulated behind the block in the cycle and appeared in large quantities in the urine. Furthermore, in the malonate-treated animal, succinate, the plasma level of which was markedly increased, appeared in large quantities in the urine. In the case of citrate and α-ketoglutarate, however, their increased rate of excretion was not accompanied by any change in their plasma levels. Thus the evidence indicated that tubular synthesis of citrate and α-ketoglutarate from the injected precursors must have occurred, just as we have visualized it occurring during the secretion of malate in the experiments reported in the present paper. Abundant confirmation of the observations of Orten and Smith (20) and Krebs, Salvin and Johnson (21) is presented in the recent comprehensive paper by Nordmann and Nordmann (2).

All these studies make it appear likely that citrate, α-ketoglutarate, fumarate, malate and oxaloacetate excretion during infusion of one of these acids occurs variously by combined processes of glomerular filtration and tubular secretion. Reabsorption may also be occurring, as with malate, but the net process appears to be tubular secretion under these circumstances. Only in the case of malate, in the study of Craig and associates (8) and the present one, has such a mechanism been shown experimentally. Similar studies with these other members of the tricarboxylic acid cycle are in progress.

It would appear that the organic aciduria of metabolic and respiratory alkalosis is also the result of tubular synthesis and secretion of citrate and α-ketoglutarate. Milne, Scribner and Crawford (22) recently reported an observation strongly supporting this concept. They found that the increased citrate excretion of rats given potassium bicarbonate was associated with a considerable rise in renal tissue citrate; in fact, they reported citrate levels comparable to those seen in kidney tissue after fluoroacetate poisoning. One would visualize the synthesis of citrate and α-ketoglutarate (and thus their urinary excretion) as being regulated primarily by intracellular pH. This in turn would be a function of potassium concentration and associated intracellular bicarbonate (23). Organic acid excretion would thus become a function of pH in the tubule cells rather than of urinary pH or general changes in acid-base balance of the body.

In support of this idea is the fact that the "condensing enzyme" in tissues shows an equilibrium state much more in favor of citrate production as pH rises (19). Evans, MacIntyre, MacPherson and Milne (4) have shown that citrate and α-ketoglutarate excretion rises in potassium-depleted man given potassium chloride even in the face of a falling urinary pH. And Orten and Smith (20) as well as Cooke and co-workers (5) have stressed the independence of organic aciduria and urinary pH. In like manner both the tubular reabsorption and secretion of malate, in the present studies, appear to be independent of urinary pH and bicarbonate excretion. For example, in one experiment during the infusion of sodium *l*-malate, urine pH gradually rose from 7.30 to 7.81 and the rate of bicarbonate excretion, from 86 to 182 μM per minute. But malate reabsorption remained relatively constant at 3.50 to 5.30 mg. per minute. Similar independence of malate secretion rate during succinate infusion has been observed.

The functions of organic acid excretion in relation to overall body economy are not completely clear. Cooke and associates (5) have suggested that organic acids may be secreted by the tubules and function as part of a fixed anion (chloride) conserving mechanism in alkalosis analogous to the fixed cation conservation mechanism that operates in acidosis. The decrease in chloride excretion that accompanied the increase in organic acid excretion in Cooke's (5) studies is suggestive of such a mechanism. However, as we have seen, the term "alkalosis" is probably too restrictive to cover the basic intracellular mechanism regulating organic acid excretion.

In addition to this function, Shorr and his associates (24) emphasized that citrate excretion appears to play an important role in the solubilization of calcium in the urine and thus the prevention of renal calcinosis. Harrison and Harrison (7) in a different approach to the problem have ob-

served in rats that the administration of Diamox® causes a profound inhibition of citrate excretion without a concomitant reduction in urinary calcium. Citrate can chelate calcium and in its absence calcium phosphate precipitates, with renal stones forming as a result. In line with the concept of citrate synthesis presented above, the action of Diamox® can be seen in terms of a reduction in intracellular citrate synthesis accompanying a reduced potassium, bicarbonate and pH in the tubule cells.

Another function of organic acid excretion appears to be related to the hormonal regulation of the menstrual cycle. Shorr, Bernheim and Taussky (25) have observed that citrate excretion is lowest during menstruation and rises in the immediate postmentrual period until, after a dip for a day or two in the midperiod, it rises to even higher rates that are sustained until the sharp decline just before the onset of the next menstruation. Evidence supporting the sex steroid control of these variations in citrate excretion derives from the fact that they can be produced at will by estradiol injection in amenorrheic females. Testosterone given to hypogonadal males also affects citrate excretion but in the opposite direction. The functional basis for these hormone-induced variations in citrate excretion is not at all understood. They may be the secondary result of a systemic change in acid-base balance or they may be the result of a steroid effect on tubular production of citrate. They occur without commensurate changes in plasma citrate levels.

The studies reported here have been mainly concerned with one of the substrates of the tricarboxylic acid cycle. To what extent these findings with malic acid relate to the general problem of organic acid excretion and its physiologic role cannot be stated with certainty at present. However, several important facts have emerged. First, this study has clearly demonstrated that at least one of the members of the tricarboxylic acid cycle can be secreted by the tubules in response to infusion of certain other members of the same cycle. Secondly, the succinoxidase enzyme system is apparently an important component of this mechanism. Thirdly, the curious and interesting fumarate "catalysis" of malate reabsorption has been described. This phenomenon suggests that elements of the cycle are involved in both the tubular secretion and reabsorption of malate.

Thus the general concept presents itself that the tricarboxylic acid cycle, usually considered as an oxidative mechanism in energy metabolism, functions also in the kidney in the synthesis, reabsorption and secretion of its own intermediates. This may be analogous to the way pyridoxal phosphate functions in amino acid transport (26) in a way quite separate from its role in intermediary metabolism of amino acids, or to the way adenosine triphosphate functions in the red cell membrane in phosphate transport (27) quite separately from its role in intracellular energy metabolism. Much additional work will be required before this hypothesis can stand or fall on the basis of solid observation.

SUMMARY

The mechanism of excretion of malic acid was studied in the dog during infusion of several tricarboxylic acid cycle intermediates including *l*-malic acid itself. With infusion of citrate, α-ketoglutarate and succinate there was glomerular filtration and net tubular secretion of malate. With each of these substrates following infusion of the succinoxidase inhibitor, malonic acid, the net tubular secretion of malate completely disappeared and glomerular filtration with net reabsorption of malate was seen instead. Thus it appears that the tubules are capable of synthesizing malate from precursors in the tricarboxylic acid cycle and secreting it into the tubular urine. In contrast to these findings, the infusion of fumarate or *l*-malate leads only to malate filtration and reabsorption. Thus the tubules are capable of both secretion and reabsorption of malate under certain definite conditions. During *l*-malate infusion there was a definite "Tm" for malate reabsorption that averaged 4 to 8 mg. per minute. During fumarate infusion the capacity of the tubules to reabsorb malate was greatly increased. This phenomenon was shown to be the result of a "catalytic" effect of fumarate on malate reabsorption. The relation of these studies to the operation of the tricarboxylic acid cycle in the kidney and the whole problem of organic acid excretion was discussed.

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STUDIES ON THE ISOLATION AND CHARACTERIZATION OF THE "RHEUMATOID FACTOR"

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Considerable evidence has been accumulated which indicates that a large proportion of the sera from patients with rheumatoid arthritis contains a factor which has the property of agglutinating particulate bodies such as red blood cells or latex particles when they are coated with γ-globulin or antibodies. Some of these reactions have been employed as diagnostic procedures and the factor or group of factors involved has been termed the "rheumatoid factor." A number of observations (1, 2) have demonstrated that the rheumatoid factor is a γ-globulin with the solubility properties of a euglobulin. More recently, ultracentrifuge experiments (3, 4) have indicated that the rheumatoid factor is a high molecular weight γ-globulin that exists in serum and plasma in the form of a complex. This complex can be visualized directly by means of the analytical ultracentrifuge in whole serum and in γ-globulin and euglobulin fractions and has a corrected sedimentation rate (s rate) of approximately 22S. Previous studies (4, 5) on the dissociation of the 22S complex by means of acid and urea have demonstrated that it is composed of two major constituents, one a 7S component and the other 19S. The latter was found to be highly active by the serological reactions for rheumatoid factor. The rheumatoid factor thus appeared to belong to the 19S class of γ-globulins which is known to include certain antibodies as well as the Waldenstrom type macroglobulins (6, 7).

Considerable purification of the rheumatoid factor has been achieved by cellulose ion exchange chromatography (8, 9), by elution from pneumococcus-antipneumococcus complexes (10), by elution from sensitized sheep cells (11), by ultrafiltration with membranes of graded porosity (12), and by preparative ultracentrifugation (4, 13), but isolation in sufficient amounts to determine the complete physical and chemical properties of the rheumatoid factor has not been reported. The observations that rheumatoid factor precipitated directly with γ-globulin (14) which had been aggregated by the procedures of isolation or heating at 63°C. for 10 minutes (3, 13) offered a method of ready concentration from large volumes of serum. Furthermore the direct visualization of rheumatoid factor in the ultracentrifuge offered a means of observing purification particularly from certain unique sera where it represented a substantial portion of the total γ-globulin.

In the present study rheumatoid factor has been obtained in a high state of purity by several different procedures. Investigations utilizing these preparations have demonstrated that rheumatoid factor is a 19S γ-globulin with chemical and immunological properties similar to those of the normal 19S class of γ-globulins.

MATERIALS AND METHODS

Sera from 4 patients with rheumatoid arthritis were employed in the isolation experiments. These were screened from approximately 100 patients with the disease and upon analytical ultracentrifugal examination contained large amounts of 22S complex as well as showing very high titers in the latex fixation and sensitized sheep cell agglutination reactions. Serum P. R. was unique in the group since it contained at least four times as much rheumatoid factor as the others and showed approximately 1,000 mg. per cent of high molecular weight complex. Serum E. P., R. P. and M. S. contained from 50 to 150 mg. per cent of 22S complex which could be visualized by direct ultracentrifugation of whole serum, euglobulin or γ-globulin fractions.

Euglobulin preparations were prepared from these sera by dilution with 12 volumes of deionized distilled water. After washing with distilled water the precipitates were dissolved in 5 per cent NaCl. Aggregated γ-globulin was prepared by heating the γ-globulin (Lederle Fraction II, 10 mg. per ml.) to 63° C. for 10 minutes. Gamma-globulin-rheumatoid factor precipitates were prepared by adding this altered γ-globulin to rheumatoid serum diluted eight times with isotonic saline. The precipitates were washed once with a large volume of saline and then dissolved in glycine-HCl buffer at pH 3 or in 4 to 6 M urea. The minimum volume was utilized which would completely dissolve the precipitates. In certain of the experiments some of the aggregates of γ-globulin were

precipitated by the addition of NaCl to the acid dissolved precipitates. Two-tenths ml. of 14 per cent NaCl was added to each 1 ml. of acid dissolved precipitate and the resulting precipitate discarded.

The euglobulin solution or dissolved γ-globulin-rheumatoid factor precipitates were layered over a sucrose or NaCl density gradient and separated in the ultracentrifuge utilizing a swinging bucket rotor. The details of this method have been published previously (13). Five-tenths to 1.5 ml. protein solution was layered over 4 ml. of sucrose or NaCl. A time of centrifugation was utilized which permitted the 19S class proteins to sediment to the lower third of the tube and leaving the 7S class proteins in the upper third. The γ-globulin aggregates packed as a pellet at the bottom of the tube. The NaCl density gradient was not as efficient as the sucrose but it proved preferable for obtaining rheumatoid factor preparations for carbohydrate analyses as well as for other analytical experiments; this was because of the difficulty of completely removing sucrose by dialysis and the deleterious effect of prolonged dialysis on the isolated preparations. In most experiments iodoacetate or parachloromercuribenzoate were added to the rheumatoid factor preparation during purification to prevent reduction of the disulfide groups in the protein. Final preparations were concentrated by ultrafiltration.

Solid γ-globulin for adsorption of the rheumatoid factor was obtained by heating Fraction II γ-globulin (Lederle) at a concentration of 40 mg. per ml. to 75° C. The resulting precipitate was washed three times with saline and the final packed precipitate suspended in a finely divided state in two volumes of saline. This material was added to rheumatoid serum. In one experiment 2 ml. of the solid suspension was added to 5 ml. rheumatoid serum and allowed to stand overnight. The precipitate was spun off and added to a second 5 ml. of serum. This was carried out with 25 ml. serum before evidence was obtained by precipitin analysis that no more activity was adsorbed by the solid γ-globulin. The precipitate was then washed three times with saline and the rheumatoid factor eluted with glycine-HCl buffer. Following concentration the eluate was layered over the NaCl density gradient and the 19S rheumatoid factor separated in the ultracentrifuge.

Analytical ultracentrifugation was carried out in a Spinco Model E machine utilizing procedures described previously (3). Free solution electrophoresis was done by the method of Longsworth employing a micro cell. Zone electrophoresis experiments were made in a starch supporting medium after first demonstrating that the rheumatoid factor was not adsorbed to the medium in water flow experiments. All electrophoresis experiments were made at pH 8.6, ionic strength 0.1, in barbital buffer.

Carbohydrate assays were carried out by the same procedures previously utilized for analyses of normal 7S and 19S γ-globulin (15). Serological determinations of rheumatoid factor were made by standard procedures (3). Precipitin tests were carried out in capillary tubes and the interphase precipitate between rheumatoid factor and γ-globulin which was heated at 63° for 10 minutes, visualized. Precipitin curves in studies on the antigenicity of the rheumatoid factor were carried out by procedures described previously utilizing the modified Folin method for analysis of specific precipitates. Five-tenths ml. of antiserum was used and the nitrogen values in Figure 9 were obtained with this volume of serum. Double-diffusion-in-agar analyses were completely comparable to those used in studies on normal 19S γ-globulin (16). Five different types of antisera were employed and material from more than one rabbit was available in each case. Complement was inactivated by heating at 56° C. for 30 minutes. Antiserum 145 was prepared against normal 19S γ-globulin. The antigen contained approximately 85 per cent 19S protein and 10 per cent 7S γ-globulin. The antibodies were primarily against the 19S constituent. Antiserum 137 was prepared against Fraction II γ-globulin which was demonstrated to be free of 19S material, and the antiserum contained no demonstrable antibodies specific for the 19S constituent. Antiserum 154 was prepared against electrophoretically isolated γ-globulin from a patient with rheumatoid arthritis. The antigen in this case contained approximately 60 per cent 7S, 15 per cent 19S, and 25 per cent 22S material. This antiserum reacted strongly with isolated rheumatoid factor even after complete absorption with 7S γ-globulin. Antiserum 169 was prepared against isolated macroglobulin S prepared from the serum of a patient with Waldenstrom's macroglobulinemia. Antiserum 200 was prepared from macroglobulin L of a different case of Waldenstrom's macroglobulinemia. Each of these antisera was used both unabsorbed and absorbed with 7S γ-globulin. The latter was obtained from a Lederle Fraction II preparation and contained no 19S material on analytical ultracentrifugation. The absorption was usually carried out by three separate additions of 7S material starting with equivalence amounts until no further precipitation was evident. The Waldenstrom macroglobulins were prepared by combined euglobulin precipitation and electrophoresis. They consisted of > 95 per cent high molecular weight protein.

RESULTS

I. Isolation from euglobulin and γ-globulin fractions

Experiments published previously indicated that the activity in the various rheumatoid tests was associated with a rapidly sedimenting component that could be directly visualized in the ultracentrifuge in certain high titer sera. Figure 1 shows the analytical ultracentrifuge pattern of whole Serum P. R. along with the γ-globulin and euglobulin fractions. Determination of the sedimentation rate demonstrated that the rapidly sedimenting peak in each case had an s rate of approximately

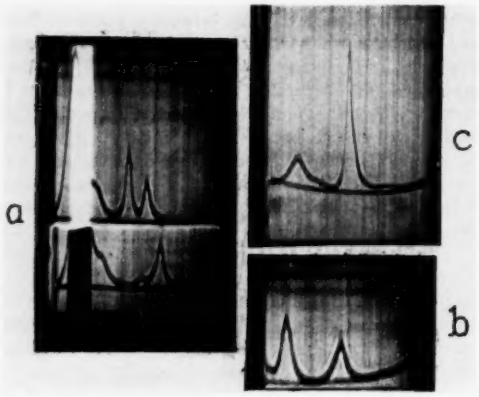

FIG. 1. ULTRACENTRIFUGE PATTERNS OF WHOLE SERUM P. R. (a) AND γ-GLOBULIN (b) AND EUGLOBULIN (c) FRACTIONS

The sedimentation is from left to right. The most rapidly sedimenting peak represents the rheumatoid factor complex. The upper pattern in (a) shows the serum after addition of 19S macroglobulin.

22. The normal 19S component is barely visible in the whole serum pattern and not at all in the euglobulin and γ-globulin fractions. The upper portion of Figure 1a is a duplicate of the lower pattern except that 19S material from a case of Waldenstrom's macroglobulinemia was added. Similar addition experiments with 19S macroglobulins also showed the higher s rate of the abnormal component in the γ-globulin and euglobulin fractions. The euglobulin fraction of this unusual serum already represented considerable purification of the rheumatoid factor and the activity per mg. of nitrogen was considerably increased. The recovery was approximately 80 per cent. In the other rheumatoid sera, the euglobulin fraction showed considerably larger amounts of contaminating protein and the normal 19S component could be seen as a major peak.

Further purification was achieved by density gradient centrifugation. The euglobulin fraction from Patient P. R. was dissolved in glycine-HCl buffer at pH 4.0 in order to dissociate the 22S complex. It then was layered immediately over a sucrose density gradient. Ultracentrifugation for 16 hours at 110,000 × G permitted the isolation of high molecular weight material at the bottom of the tube free from 7S components which remained in the upper third of the tube. Figure 2b shows an ultracentrifuge pattern of the active

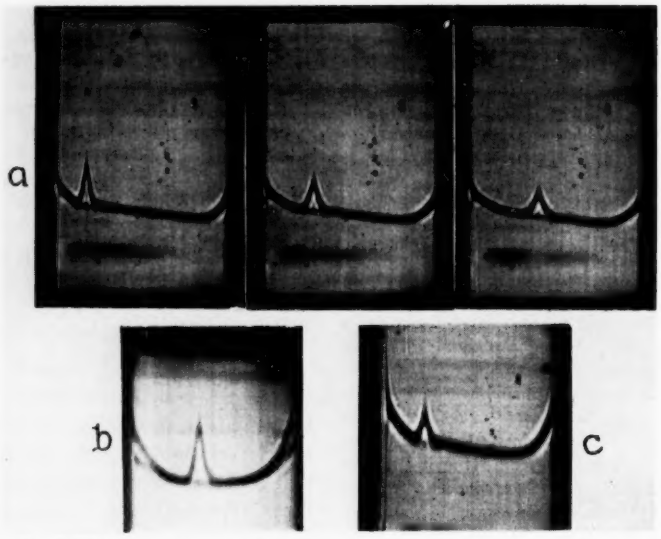

FIG. 2. ULTRACENTRIFUGE PATTERNS OF ISOLATED RHEUMATOID FACTOR PREPARATIONS

The upper patterns (a) are of Preparation II at 12, 20 and 26 minutes at 52,000 rpm. The lower patterns (b) of Preparation I and (c) of Preparation VII are at 28 and 20 minutes, respectively.

material isolated from the lower part of the density gradient tube. It shows a single major peak with an s rate of 18.7S. Figure 3 shows the electrophoretic pattern obtained for this preparation by zone electrophoresis in a starch supporting medium. The pattern of the original serum is also shown. The results of activity analyses are indicated and show the marked purification achieved in terms of activity per mg. of protein. The mobility of the activity is similar in the isolated preparation to that in the whole serum with possibly a slight shift to the faster migrating portion of the γ-globulin. Although this preparation appeared quite homogeneous by zone electrophoresis, experiments by moving boundary electrophoresis indicated one peak that was too broad to represent a single component. Table I shows the activity per mg. of this preparation (Preparation I) and other similar preparations. Unfortunately, these tests are only semiquantitative, and the exact activity per mg. is not satisfactorily established. In some experiments the euglobulin or γ-globulin was centrifuged directly without dissociation of the 22S complex with acid. Some dissociation appeared to occur with this procedure alone but it was not always complete and since no detectable loss of activity was observed following acid dissociation, the euglobulin was usually dissolved in acid buffer prior to zone centrifugation. All of the titers by the latex test in Table I are after 1.5 hours incubation. Higher titers were obtained following overnight standing.

TABLE I

Serological activity of eight different preparations of rheumatoid factor homogeneous in the ultracentrifuge

Preparations	Latex fixation	Sensitized sheep cell agglutination	Sensitized sheep cell agglutination + sheep serum
1 mg./ml. conc.			
I P. R. euglob.*	1/10,240	1/640	1/5,120
II P. R. 63° prec.*	1/20,480	1/1,280	1/5,120
III R. P. euglob.†	1/10,240	1/5,120	1/5,120
IV R. P. 63° prec.*	1/10,240	1/5,120	1/10,240
V R. P. Solid eluate*	1/10,240	1/5,120	1/5,120
VI E. P. 63° prec.‡	1/10,240	1/320	1/5,120
VII M. S. 63° prec.*	1/5,120	1/640	1/5,120
VIII E. L. 63° prec.*	1/10,240	1/2,560	1/10,240

* Precipitate dissolved in acid.
† No acid employed in this isolation.
‡ Precipitate dissolved in urea.

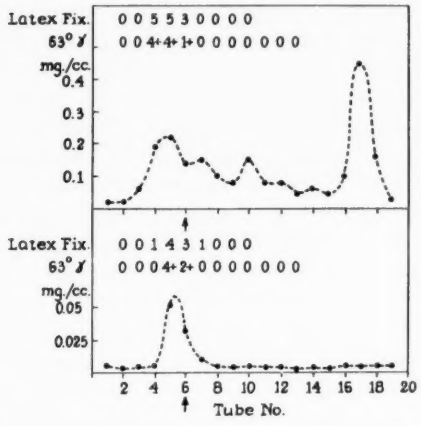

FIG. 3. ZONE ELECTROPHORETIC PATTERN OF SERUM P. R. (UPPER CURVE) AND THE PATTERN OF PREPARATION II ISOLATED FROM THIS SERUM (LOWER CURVE)

The separation was carried on the same starch block and the segments correspond in the two curves. The activity by the latex fixation test is indicated by tube numbers of serial dilutions giving a positive reaction. The precipitin reactivity with heated γ-globulin is also indicated.

Isolation procedures of this type from euglobulin and γ-globulin fractions cannot be considered a method for the complete isolation of the rheumatoid factor because of contamination with normal 19S γ-globulin. The latter has similar properties and is carried along. However Serum P. R. contained such a uniquely large amount of rheumatoid factor as indicated by the ultracentrifuge patterns of the euglobulin and γ-globulin fractions, that contamination by normal 19S γ-globulin must have been less than 10 per cent. This is not true of the preparations from the sera of Patients R. P. and E. P. where the contamination was significant although not sufficient to be reflected in the titers of the serial dilution tests shown in Table I.

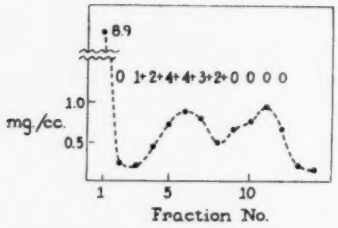

FIG. 4. CURVE OBTAINED BY PROTEIN ANALYSES ON FRACTIONS OF DISSOLVED SPECIFIC PRECIPITATES, SEPARATED BY DENSITY GRADIENT ULTRACENTRIFUGATION

Fraction 1 is from the bottom of the centrifuge tube; Fraction 14 is from the top. The peak in Fractions 4 through 7 represents the 19S rheumatoid factor. The activity in the precipitin reaction is indicated above the curve.

II. Isolation from specific precipitates

In order to eliminate normal 19S γ-globulin from isolated fractions of rheumatoid factor the specific property of this material to precipitate with aggregated γ-globulin was utilized for complete purification. The rheumatoid factor was precipitated from whole serum and euglobulin or γ-globulin fractions. The precipitates were dissolved in glycine-HCl buffer, layered over a density gradient, and separated in the ultracentrifuge. Figure 4 illustrates the pattern obtained by analyses of fractions taken from the density gradient tube at the end of the experiment. Three main peaks are visible. One, which comes from the pellicle at the bottom of the tube, represents primarily aggregated γ-globulin. The second peak which was the active fraction represented 19S rheumatoid factor and the third peak in the upper portion of the tube consisted of material with an s rate between 6 and 12 (usually primarily approximately 7S). The latter was inactive in the various serological reactions and originated primarily from the lower molecular weight portion of the soluble 22S complex from the original serum (4, 5). Combination of the fractions from the 19S peak (Tubes 4 through 7 in the experiment shown in Figure 4) furnished highly purified preparations of rheumatoid factor. Figures 2a and 2c illustrate ultracentrifuge patterns of two of these preparations. The activity per mg. of some of these preparations is shown in Table I. This method made it possible to isolate rheumatoid factor from relatively weakly positive sera. Preparation VII was obtained from Serum M. S. which had only approximately one-tenth the activity of Serum P. R. Recovery of activity was difficult to determine from activity analyses but by indirect calculation from the amount of 22S component observed in the original serum a value close to 80 per cent could be obtained. In some experiments the dissolved precipitates were partially fractionated prior to centrifugation by precipitation of the aggregated γ-globulin from the acid solution by raising the ionic strength with NaCl. Urea (4 to 6 M) was also used to dissolve the specific precipitates prior to centrifugation (Preparation VI) and results similar to those with acid were obtained. With both the urea and acid experiments, ultracentrifugation was begun within a few minutes after dissolving the precipitates and the rheumatoid factor then sedimented out of these solutions into the density gradient. As a result it was exposed to urea or acid only for a relatively short time.

III. Isolation by adsorption on solid γ-globulin

Previous observations (13) indicated that solid γ-globulin prepared by heat precipitation under controlled conditions adsorbed the rheumatoid factor which then could be eluted by means of acid buffers or urea. Analyses of these eluates indicated that they consisted of from 50 to 70 per cent 19S material. Removal of the eluting solvent by dialysis gave highly active saline solutions but relatively poor recovery because of considerable precipitation. Direct ultracentrifugation in the density gradient of concentrated eluates in acid avoided precipitation and resulted in good recovery of 19S rheumatoid factor in highly purified form. One preparation, the activity of which is listed in Table I, consisted of 96 per cent of one component by ultracentrifuge analysis. This method had advantages over the dissolved precipitate procedure because the absence of the dissolved aggregates of γ-globulin facilitated the purification in the ultracentrifuge. The major limitation was in the fact that some preparations of solid γ-globulin removed only slight activity from the original serum while others removed all activity. Sera from different patients also varied in respect to the removal of activity.

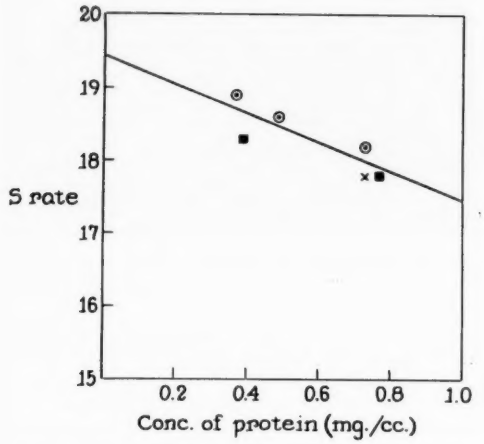

FIG. 5. THE S VS. C PLOT FOR SEVERAL PREPARATIONS OF ISOLATED RHEUMATOID FACTOR SHOWING THE SEDIMENTATION RATE AT INFINITE DILUTION

By the method of least squares, $y = -1.97 \times + 19.44$. The different symbols represent different preparations.

IV. Physical and chemical properties

Figure 5 shows a plot of the s *vs.* c relationship for several preparations of isolated rheumatoid factor examined in the ultracentrifuge. The extrapolated line obtained by the method of least squares shows after correction for the buffer solvent that $s^{\circ}_{20,w} = 19.4S$.

Five samples of isolated rheumatoid factor prepared from acid dissolved precipitates were studied by free solution electrophoresis. All of these preparations were found to be heterogeneous. In two instances a single peak was observed but this was broader than would have been expected for a single component (Figure 6a). Reversible boundary spreading experiments confirmed this heterogeneity. In three instances a second peak could be seen on the faster side of the main peak. This is illustrated in Figure 6b. Both peaks were broader than expected for single components. Ultracentrifugal analysis of these same preparations demonstrated that the second peak also represented 19S protein and was not due to contaminants. In one experiment the second peak made up 18 per cent of the total protein by electrophoresis but by ultracentrifugal analysis there was less than 5 per cent of proteins other than those in the 19S peak. In addition, strong evidence was obtained that the faster migrating material was rheumatoid factor in that it again precipitated with aggregated γ-globulin when this was added to the isolated preparation. In several experiments all detectable protein in the isolated fraction reprecipitated on the addition of aggregated γ-globulin. The mobility of the major peak ranged from 1.1 to 1.5×10^{-5} in these experiments.

Table II shows the results of nitrogen and carbohydrate analyses on three different prepara-

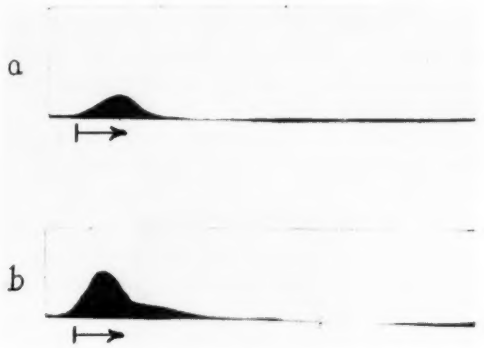

FIG. 6. FREE SOLUTION ELECTROPHORESIS PATTERNS OBTAINED ON TWO PREPARATIONS OF ISOLATED RHEUMATOID FACTOR (UPPER, PREPARATION II; LOWER, PREPARATION VIII)

Photographs taken at approximately two hours at field strength of approximately 6 volts per cm.

TABLE II

Carbohydrate content of several preparations of isolated rheumatoid factor *

Protein	N	Hexose	Hexosamine	Sialic acid	Total carbohydrate	Hexosamine/ Hexose
	%	%	%	%	%	
Norm. γ-glob. (7S)	15.64	1.22	1.14	0.22	2.58	0.94
Norm. γ-glob. (19S)	14.47	5.20	2.9	1.70	9.80	0.55
Rh. f. Prep. II	14.54	5.40	2.9	1.80	10.10	0.55
Rh. f. Prep. IV		5.3	2.0	1.70		
Rh. f. Prep. V		5.1				

* Previous results for normal 7S and 19S γ-globulin are also indicated.

tions of isolated rheumatoid factor. Preparation II was analyzed in considerable detail and the figures illustrated were obtained from duplicate assay. The experimental error of these methods has been described previously (15). Hexosamine and sialic acid values on Preparation IV were the results of single determinations. Insufficient material was available to check the one low hexosamine figure. Numerous other studies on the hexose content of different preparations gave similar results. The carbohydrate content of the rheumatoid factor was very close to that previously reported for the normal 19S fraction of γ-globulin (15) which is shown in Table II for purposes of comparison.

Mercaptoethanol and other sulfhydryl reagents were found to dissociate the rheumatoid factor in a manner similar to that reported for Waldenstrom macroglobulins (17) and the cold agglutinins (7). Some of these results have been published previously (4). Activity in the latex fixation, sensitized sheep cell agglutination and precipitin tests was lost with the dissociation. Figure 7 shows the ultracentrifugal pattern of a purified preparation before and after treatment with 0.1 M mercaptoethanol. The major peak showed a shift in s rate from approximately 19S to approximately 7S.

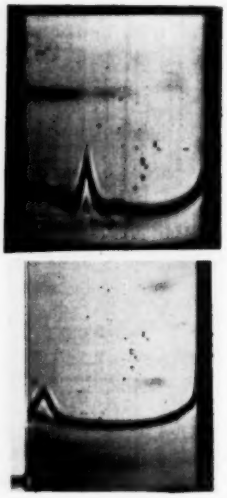

FIG. 7. ULTRACENTRIFUGE PATTERNS OF ISOLATED RHEUMATOID FACTOR BEFORE (UPPER) AND AFTER (LOWER) DISSOCIATION WITH MERCAPTOETHANOL.

Patterns at 28 minutes in each case.

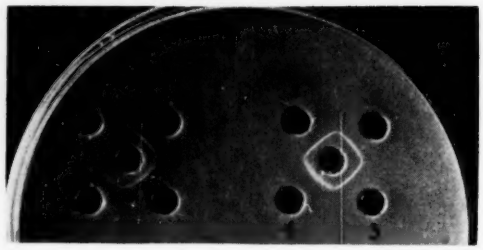

FIG. 8. OUCHTERLONY PLATES SHOWING REACTION OF VARIOUS ANTISERA WITH ISOLATED RHEUMATOID FACTOR

The antisera used in both experiments were the same except that in the left series each antiserum was absorbed with 7S γ-globulin. The rheumatoid factor is in the middle well in both instances. The antisera beginning at the upper left and proceeding clockwise are as follows in both instances: *1*) As. 137 against 7S γ-globulin; *2*) As. 145 against normal 19S γ-globulin; *3*) As. 154 against rheumatoid factor γ-globulin; *4*) As. 169 against an isolated Waldenstrom macroglobulin.

V. Immunological studies

Various modifications of the Ouchterlony procedure indicated a close immunological relationship between the isolated rheumatoid factor, normal 19S γ-globulin and isolated macroglobulins prepared from cases of Waldenstrom's macroglobulinemia. Figure 8 illustrates one of these experiments. Preparation IV of isolated rheumatoid factor was placed in the center well and reacted with four different antisera, both unabsorbed and after absorption with 7S γ-globulin. A single major line which fuses is visible with the unabsorbed antisera. Absorption removed the line with antiserum 137 which contained primarily antibodies to 7S γ-globulin. The absorbed antisera reacted with the rheumatoid factor in a single line and little difference in reactivity could be detected between the antisera against normal 19S, rheumatoid factor or macroglobulin. Control experiments with isolated normal 19S γ-globulin gave almost identical results with these same antisera.

When experiments were carried out with parallel wells and isolated rheumatoid factor compared directly with normal 19S γ-globulin, single lines were observed with the different antisera and the line for normal 19S protein fused with that for rheumatoid factor without spur formation. In these experiments both the normal 19S line and the rheumatoid factor line showed curvature to-

ward the antigen well indicating, as demonstrated by Korngold and Van Leeuwen (18), that the observed reaction was due to a high molecular weight component.

Figure 9 illustrates precipitin curves of these same absorbed antisera with isolated rheumatoid factor as well as with isolated Waldenstrom macroglobulins and normal 7S γ-globulin. Figure 9a shows the curves obtained with rheumatoid factor, macroglobulin L and macroglobulin B with antiserum to normal 19S γ-globulin. The rheumatoid factor reacts slightly more strongly. A similar relationship is seen in Figure 9b where antiserum to rheumatoid factor was employed. Figure 9c shows that rheumatoid factor reacts more strongly with antiserum to macroglobulin S than do heterologous macroglobulins B and L. However, homologous macroglobulin L reacts most strongly with antimacroglobulin L (Figure 9d). It appeared clear that rheumatoid factor reacted strongly per unit of nitrogen as compared with isolated Waldenstrom macroglobulins against macroglobulin antisera absorbed with 7S γ-globulin. In the course of these experiments, three different antisera to rheumatoid factor γ-globulin were studied. None of these showed any reaction with rheumatoid factor following absorption with normal γ-globulin rich in 19S components.

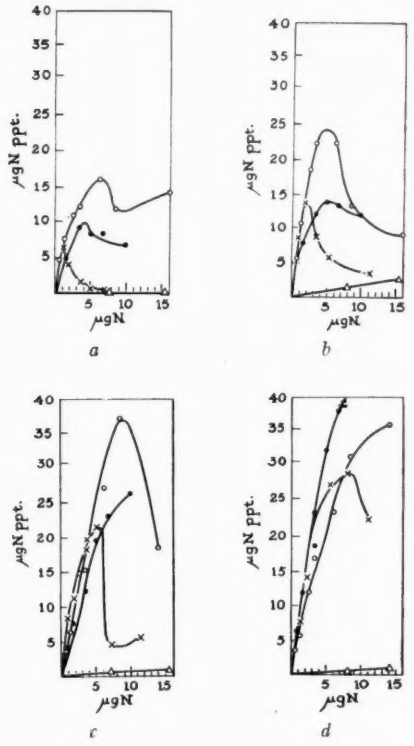

FIG. 9. PRECIPITIN CURVES OF ISOLATED RHEUMATOID FACTOR (○—○), MACROGLOBULIN L (●—●), MACROGLOBULIN B (×—×) AND 7S γ-GLOBULIN (△—△) WITH VARIOUS ANTISERA ABSORBED WITH 7S γ-GLOBULIN

a) Antiserum 145 against normal 19S γ-globulin.
b) Antiserum 154 against rheumatoid factor γ-globulin.
c) Antiserum 169 against macroglobulin S.
d) Antiserum 200 against macroglobulin L.

DISCUSSION

The use of sera containing very high titers in the serological tests and uniquely large amounts of rheumatoid factor as demonstrated directly in the ultracentrifuge made it comparatively easy to isolate this factor in a highly purified state. Serum P. R. which was used extensively in this study contained approximately 10 mg. per ml. of rheumatoid factor complex and the γ-globulin fraction consisted of approximately 40 per cent of this material. The high concentration of rheumatoid factor in this serum made isolation possible directly from euglobulin and γ-globulin fractions with only slight contamination with normal 19S class proteins. With other sera this contamination proved substantial and the specific property of precipitation with aggregated γ-globulin had to be utilized for satisfactory purification. The methods used for isolation all utilized preparative ultracentrifugation for final purification and since the high molecular weight property was somewhat unique the isolated fractions consisted of close to 100 per cent rheumatoid factor. This was indicated both by the homogeneity of the analytical ultracentrifuge patterns and by the reactivity and utilization of all of the protein in the precipitin tests with aggregated γ-globulin. Despite this evidence of biological homogeneity, free solution electrophoresis experiments indicated considerable heterogeneity and it was clear that a group of different proteins was involved. The heterogeneity was similar to that described for γ-globulin itself (19). This was to be expected since activity curves on whole serum separated by zone electrophoresis indicated that the activity peak was too

broad to represent a single protein species. In addition, it is known that in serological tests employing human γ-globulin the latter reacts with more rheumatoid factor protein than does rabbit γ-globulin in tests where it is the reactive substance (20, 21). The picture resembles that known for the usual mixtures of species specific and cross-reacting antibodies. Recently, Lospalluto, Lewis and Ziff have succeeded in separating at least two of these factors by ion exchange chromatography (8). In the present study, absorption experiments were also carried out with antigen-antibody complexes by the method of Vaughan (20) and with finely dispersed solid rabbit γ-globulin. Only part of the isolated rheumatoid factor was absorbed leaving material that reacted strongly with human γ-globulin. In addition, one macroglobulin preparation was isolated from a sarcoid serum that showed no activity in the sensitized sheep cell test but high titers by the tests employing human γ-globulin. The accumulated evidence points to the existence of multiple, closely related macroglobulins which have the common property of reacting with human γ-globulin.

Suggestive evidence that the rheumatoid factor contains considerable carbohydrate was obtained by a number of workers prior to its isolation (20, 22, 23). The present observations indicate approximately 10 per cent carbohydrate made up of hexose, hexosamine and sialic acid. The analytical values are very close to those previously obtained for normal 19S γ-globulin and for certain macroglobulins from the sera of patients with Waldenstrom's macroglobulinemia.

The immunological results with the isolated rheumatoid factor also indicate a close relationship to normal 19S γ-globulins and pathological macroglobulins. Antisera to crude rheumatoid factor preparations did not show antibodies specific for this type of protein and these antisera could be absorbed completely by normal proteins. The possibility must be considered that rheumatoid factor exists in normal serum and is a component of normal 19S γ-globulin, thus accounting for some cross-reactions between different antisera. However, quantitative studies indicated that the close immunological relationship between rheumatoid factor, normal 19S γ-globulin and pathological macroglobulins could not be accounted for by small amounts of rheumatoid factor in the latter preparations. All the evidence pointed to the fact that these 19S proteins with different biological properties behaved very similarly as antigens. Future work may indicate fine differences between rheumatoid factor and normal 19S proteins as has already been demonstrated between different Waldenstrom macroglobulins (24). Difficulties in the complete isolation of adequate amounts of normal 19S proteins hampered final resolution of this question. In addition, the secondary reaction of rheumatoid factor with soluble and insoluble antigen-antibody complexes (13, 20) obscured certain of the quantitative interpretations particularly in regions of antigen excess for rheumatoid factor.

Considerable evidence has accumulated indicating that the 19S class of γ-globulins in normal and certain pathological sera are made up of a group of different antibodies. Certain of the isoagglutinins, the saline agglutinating rh antibodies, certain of the Wasserman antibodies, heterophile antibodies, cold agglutinins, certain of the typhoid agglutinins and many other antibodies have been localized in this fraction (6, 7, 25, 26). The Waldenstrom macroglobulins appear to be exceptions and seem to bear a relationship to the 19S class of γ-globulins similar to that of the myeloma proteins to the major 7S class of γ-globulins. Since the rheumatoid factor, on the basis of the ultracentrifugal data, the carbohydrate content, the ready dissociation with mercaptoethanol and the immunological results, is closely related to the 19S class of γ-globulins, the possibility is raised that it represents an antibody. Final proof of such a hypothesis must await clear elucidation of the antigen involved. Certain evidence suggests that ordinary γ-globulin of the 7S variety is itself the antigen. No other protein has yet been found that will react with the rheumatoid factor in a similar fashion. This reaction is observed in the soluble complex in rheumatoid arthritis sera, in the precipitin reaction with aggregated γ-globulin, in the specific adsorption on solid γ-globulin, and in the agglutination of red cells coated with incomplete antibodies. In the latter reaction the rheumatoid factor acts in a manner analogous to Coombs sera or rabbit antibody to γ-globulin and sera containing rheumatoid factor actually have been utilized for this purpose (27).

SUMMARY

Highly active purified preparations of rheumatoid factor were obtained from selected sera showing high titers in the latex fixation and sensitized sheep cell agglutination reaction. Procedures of density gradient ultracentrifugation were utilized for isolation from euglobulin fractions, from dissolved specific precipitates produced by the addition of aggregated γ-globulin to rheumatoid serum and from eluates of solid γ-globulin to which rheumatoid factor had been adsorbed.

The isolated preparations were homogeneous in the analytical ultracentrifuge and showed a corrected sedimentation rate of approximately 19S. Dissociation into smaller subunits was produced readily with mercaptoethanol with simultaneous loss of activity. Free solution electrophoresis showed the preparations to be primarily γ-globulin with evidence of heterogeneity. The accumulated evidence indicated that the rheumatoid factor consists of multiple, closely related macroglobulins.

Carbohydrate analyses showed a total content of approximately 10 per cent consisting of hexose and hexosamine sugars and sialic acid. The results were very similar to those previously obtained for normal 19S γ-globulin. Immunological studies also indicated a close relationship to this normal fraction.

The question of whether the rheumatoid factor represents an antibody is discussed. The close relationship to the broad 19S class of γ-globulins which are known to contain a variety of antibodies is an additional point in favor of this hypothesis although it remains to be proven.

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THE ELECTRICAL POTENTIAL DIFFERENCE GENERATED BY THE LARGE INTESTINE: ITS RELATION TO ELECTROLYTE AND WATER TRANSFER *

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Studies of the physicochemical forces involved in the transfer of ions and water across biological membranes have received great impetus from the work on the isolated frog skin by Ussing, Zerahn and Koefoed-Johnson (1, 2). This type of approach would help to define the mechanisms involved in the transfer of fluid and electrolyte across the intestine *in vivo*. A previous study on the large intestine *in vivo* has stressed the striking concentration gradients developed between the lumen and the plasma (3). Evaluation of the driving forces for electrolyte and water transfer across the large intestine has not been possible, however, in the absence of measurements of transfer rates and electrical potential.

Recent studies on the isolated large intestine of the guinea pig and frog have indicated that the absorption of sodium is active, and that of chloride is passive (4, 5). The maintenance of an electrical potential gradient was found to be dependent upon active sodium transport. However, since the intestine can secrete (6) as well as absorb, even these studies provide limited information.

In the experiments reported here, the electrical potential difference across the large intestine of the anesthetized dog was measured. Simultaneously the net transfer of water, sodium, potassium, chloride and bicarbonate between the lumen and the blood were determined. The unidirectional movements of sodium and chloride were calculated from the rate of absorption of their radioisotopes. By correlating the electrical potential difference with observed chemical events, it was possible to obtain a more precise analysis of some of the forces influencing ionic and water transfer across the large intestine. Information was obtained which clearly demonstrates at least two active transport systems: sodium absorption and bicarbonate secretion.

* A preliminary report of this work was presented at the National Meeting of the American Federation for Clinical Research, Atlantic City, N. J., May, 1958.

METHODS

Thirteen healthy mongrel dogs weighing from 9 to 17 Kg. were used. After anesthetization with 20 to 25 mg. per Kg. of pentobarbital sodium the abdomen was entered through a small midline incision. A segment of large intestine, 4 cm. distal to the caecum and 10 to 15 cm. in length, was divided and flared cannulae carefully tied into each end. Care was taken to keep the blood supply intact. The cannulae were exteriorized through two small stab wounds and the abdomen closed. The intestinal segment was rinsed clean with isotonic saline warmed to 37° C. An electrolyte solution from a reservoir was recirculated through the intestinal segment using plastic tubing and a Bowman infusion pump. The solution in the reservoir was maintained at 39° C. The initial total volume of perfusion solution varied from 100 to 200 ml. depending on the size of the intestinal segment, and the flow rate ranged from 5 to 10 ml. per minute. A modified Krebs-Henseleit solution was used with 100 mg. per cent of glucose and 1.25 mEq. of calcium per L. (7). The concentrations of Na^+, Cl^-, K^+ and HCO_3^- in this solution were 138, 120, 5 and 22 mEq. per L., respectively. Penicillin (8 mg. per cent) and streptomycin (16 mg. per cent) were added to minimize bacterial contamination. Phenol red and radioactive isotopes were added to the perfusion fluid as described later.

The electrical potential difference across the wall of large intestine was detected with a pair of calomel electrodes and continuously recorded with a modified Brown-Minneapolis Honeywell recording potentiometer with an input impedence of one megohm. Two pieces of large bore polyethylene tubing (PE 320) filled with 1 M KCl and 3 per cent agar served as bridges from the calomel electrodes to the luminal solution and the peritoneal surface of the intestinal segment. The junction potential was less than 1 mV. in all instances. During the experiment an additional potential difference was measured between the luminal solution and circulating venous blood in the vena cava. In many instances the potential difference across the intestinal wall showed rhythmical phasic variations: The mean potential was then determined by planimetry. These variations in electrical potential will be the subject of a forthcoming report.

Each experiment lasted four to seven hours and hourly samples of 5.0 ml. were removed from the lumen. In

order to maintain volume, each sample was replaced with five ml. of the original solution and the resultant change in solute concentrations calculated. Chemical determinations of Na^+, K^+, Cl^- and HCO_3^- were done on each hourly sample. During each experiment, two samples of plasma were analyzed similarily. Na^+ and K^+ were determined by flame photometry using lithium as an internal standard, Cl^- by the method of Cotlove, Trantham and Bowman (8), and HCO_3^- by the method of Van Slyke and Neill. Osmolality of the plasma and luminal content were determined by the freezing point depression method (9).

Phenol red (0.004 per cent) was added to the original solution, and the optical density of each hourly sample was determined at 550 mμ after a 15-fold dilution with 0.1 N NaOH (10). At the end of each experiment, the amount of phenol red left in the perfusate and saline washings was determined spectrophotometrically. Recovery of the dye was 97 ± 2 per cent, and this slight loss justifies its use as an indicator of volume change. The net water flux was calculated with the equation:

$$\text{Net water flux} = \frac{V_0 - \frac{D_0}{D_1} \cdot V_0}{t} \qquad 1)$$

where t is the time in hours, D_0 is the initial optical density, D_t the optical density at the end of the hourly period, and V_0 the volume of the initial solution.

The net flux (M_{net}) of each ion was calculated by using the equation:

$$M_{net} = \frac{V_0C_0 - V_tC_t}{t} \qquad 2)$$

where V_0 and C_0 are the total volume and concentration at the beginning of any hourly period, and V_t and C_t the volume and concentration at the end of that period. A positive net flux denotes movement from the lumen to the blood.

Trace amounts of Na^{24} and Cl^{36} were added to the original perfusion solution and the influx of these ions from lumen to blood determined by the rate of disappearance of their radioisotopes. The initial sample was taken one-half to one hour after perfusion was begun. The Na^{24} disintegration rate was determined on each sample in a well-type scintillation counter without interference from the beta particles of Cl^{36} which were absorbed by the glass wall of the vial containing the sample. The Na^{24} was then allowed to decay and the Cl^{36} distintegration rate counted with a Geiger-Muller tube after duplicate 1 ml. samples were dried on filter paper bonded to copper discs. A minimum of 10,000 disintegrations were counted and the activities were at least two to three times background.

The influx (M_i) of Na^+ and Cl^- from lumen to blood was calculated with the equation:

$$M_i = \frac{r_0V_0 - r_tV_t}{\bar{r}} \cdot \bar{C} \qquad 3)$$

where r_0 is the absolute cpm per ml., V_0 the volume at the beginning of any hourly period, r_t and V_t are these values at the end of that period, $\bar{r}$ the mean cpm per ml., and $\bar{C}$ the mean concentration of the ion in the perfusate.

In order to justify the use of Equation *3*, the following assumptions were made: First, the isotopic movement from lumen to blood is unidirectional, since the activity of Na^{24} and Cl^{36} in the blood was found to be indistinguishable from background. Second, that the rate of change in concentration and isotopic activity of Na^+ and Cl^- in the luminal fluid was linear with time. Their arithmetic mean was used since calculations made on the basis of an exponential decline gave similar results, and the change in concentrations was less than 2 per cent and the change in isotopic activity less than 10 per cent during each sampling period. Third, the kinetics of Na^+ and Cl^- transfer across the intestinal epithelium can be described in terms of two compartments: lumen and plasma.

The outflux (M_o) of Na^+ and Cl^- from blood to lumen was obtained with the equation:

$$M_o = M_i - M_{net}. \qquad 4)$$

The intestinal segment was weighed and measured at the end of the experiment and the flux arbitrarily expressed as microequivalents (μEq.) per Gm. of wet tissue per hour. Water movement was expressed as ml. per Gm. of tissue per hour.

RESULTS

Electrical

The electrical potential difference across the epithelium of the large intestine ranged in different experiments from 10 to 40 mV. In all instances the lumen was negative with respect to the peritoneal surface. The potential difference between the luminal solution and circulating blood was the same when the KCl agar bridge on the peritoneal surface of the intestine was placed in the vena cava. In some instances, rhythmical phasic variations in the potential were noted to occur with a periodicity of 3 to 5 minutes, duration of 15 seconds to 2 minutes, and amplitude of + 1 to + 20 mV. In one dog with a Thiry-Vella loop of colon, the potential between the lumen and the venous blood was noted to vary from 20 to 50 mV. over a six month period. The potential difference across the wall of the small intestine and gall bladder was measured by the technique described, using the modified Krebs-Henseleit solution. The absence of an appreciable potential difference across these organs emphasizes the significance of the observed potential in the colon.

TABLE I

Net rates of H_2O and electrolyte transfer during absorption *

H_2O	HCO_3^-†	K^+†	Na^+†	Cl^-†	P.D.
ml./Gm./hr.					
+0.42‡ (±0.11)§	+6.0 (±3.6)	−2.5‡ (±1.2)	+61.0 (±14.5)	+53 (±12.7)	19 mV.

* Eleven dogs, 55 one hour sampling periods.
† Values for HCO_3^-, K^+, Na^+ and Cl^- expressed as μEq. per Gm. of intestinal segment per hour.
‡ (+) denotes movement from lumen to blood; (−) denotes movement from blood to lumen.
§ ± = standard deviation.

Chemical

Two patterns of electrolyte and water transfer were observed. The first pattern (Tables I and II) was observed in 11 dogs and was characterized by the net absorption of water, sodium and chloride into the blood. The concentration of sodium in the luminal fluid was always less than that observed in the plasma. Thus sodium was absorbed against both a concentration and an electrical gradient. The absorption of chloride into the blood was in the direction of its electrical gradient but against its concentration gradient. The net transfer of potassium was from blood to lumen. Like chloride it moved with the electrical but against its chemical gradient. In the original perfusion solution the ratio of Cl^- to HCO_3^- was 6:1, but these ions were absorbed in the ratio of 9:1 (range, 6:1 to 20:1). The net effect of the ionic movements was an alteration of the electrolyte pattern of the luminal fluid, with sodium and chloride concentrations decreasing, and potassium and bicarbonate increasing. This concentration pattern has been observed previously (3).

The second pattern was observed in the two remaining dogs (Table III), and was characterized by a profuse bicarbonate secretion into the lumen with an accompanying net secretion of water, sodium and potassium. In this instance, bicarbonate was the predominant ion being transported against a chemical and electrical potential

TABLE II

A representative experiment showing net rates of H_2O and electrolyte transfer during absorption

Time	H_2O	HCO_3^-*		K^+*		Na^+*		Cl^-*		P.D.
hr.	*ml./Gm./hr.*†	*μEq./Gm./hr.*	*mEq./L.*	*μEq./Gm./hr.*	*mEq./L.*	*μEq./Gm./hr.*	*mEq./L.*	*μEq./Gm./hr.*	*mEq./L.*	*mV.*
0			22.1		6.0		138		120	16
0–1	+0.85‡	+ 7.1	23.9	− 3.3‡	7.4	+ 99.5	139	+109	119	16
1–2	+0.43	+ 3.8	24.9	− 0.9	8.1	+ 97.0	133	+ 57.0	118	17
2–3	+0.64	0	25.4	− 2.8	9.5	+ 86.0	132	+ 82.0	117	20
3–4	+0.57	+ 9.5	27.8	− 6.6	11.7	+ 81.0	131.5	+ 73.0	116	22
4–5	+0.58	+ 4.2	30.6	− 4.7	13.6	+ 64.0	131	+ 62.0	115	24
5–6	+0.53	+ 9.0	31.3	− 4.3	15.8	+ 80.0	129	+ 69.0	113	26
	+3.40	+33.6		−22.6		+507.5		+452.0		

* Plasma values of HCO_3^-, K^+, Na^+ and Cl^- were 22.0, 4.1, 149 and 118 mEq. per L., respectively.
† Net weight of tissue, 21.2 Gm.; length, 12.2 cm.
‡ (+) denotes movement from lumen to blood; (−) denotes movement into lumen.

TABLE III

An experiment showing net rates of H_2O and electrolyte transfer during secretion

Time	H_2O	HCO_3^-*		K^+*		Na^+*		Cl^-*		P.D.
hr.	*ml./Gm./hr.*†	*μEq./Gm./hr.*	*mEq./L.*	*μEq./Gm./hr.*	*mEq./L.*	*μEq./Gm./hr.*	*mEq./L.*	*μEq./Gm./hr.*	*mEq./L.*	*mV.*
0			25.8		5.9		149		129	25
0–1	−0.42‡	− 62.0	32.9	− 7.0	6.6	− 32	146	+ 7‡	122	25
1–2	−0.57	− 49.0	36.2	− 7.2	7.0	− 43	145	− 3	117	23
2–3	−0.13	− 23.0	39.1	− 7.2	7.9	− 23	145	+ 7	115	20
3–4	−0.05	− 20.0	42.9	− 5.5	8.9	− 20	142	− 1	114	22
	−1.17	−154.0		−26.9		−118		+10		

* Plasma levels of HCO_3^-, K^+, Na^+ and Cl^- were 24.1, 3.8, 150 and 116 mEq. per L., respectively.
† (+) denotes movement from lumen to blood; (−) denotes movement from blood to lumen.
‡ Net weight of tissue, 23.5 Gm.; length, 12.0 cm.

TABLE IV

Chloride flux ratios

Expt.	No. of one hour flux periods	$\frac{[Cl^-_{plasma}]}{[Cl^-_{lumen}]}$	$\frac{M_1}{M_0}$*	Observed† ratio	Calculated† ratio	$\frac{\text{Observed}}{\text{Calculated}} \times 100$
						%
1	7	$\frac{113}{118}$	$\frac{103}{30}$	3.3	2.7	123
2	6	$\frac{120}{113}$	$\frac{44}{9}$	5.0	2.4	200
3	5	$\frac{114}{127}$	$\frac{63}{18}$	3.1	2.2	140
4‡	6	$\frac{116}{129}$	$\frac{52}{46}$	1.2	2.4	50
5‡	4	$\frac{115}{117}$	$\frac{28}{22}$	1.3	2.5	50

* M_1 is influx (lumen to blood); M_0 is outflux (blood to lumen). Flux expressed as μEq. per Gm. per hour.

† Observed ratio calculated from $\frac{M_1}{M_0} \cdot \frac{[Cl^-_{plasma}]}{[Cl^-_{lumen}]}$; calculated ratio from antilog $\frac{E(mV.)}{60}$.

‡ Experiments 4 and 5 characterized by net secretion of water into the lumen, Experiments 1, 2 and 3 by net absorption.

gradient. Again chloride was absorbed in the direction of its electrical gradient. Both sodium and potassium entered the lumen along their electrical gradient, but the ratio of sodium to potassium secreted into the lumen was 5:1 as compared to a much higher ratio in the plasma. This demonstrates that even with secretion of bicarbonate into the lumen, the concentration pattern in the luminal fluid is still that of a decrease in sodium and chloride and an increase in potassium and bicarbonate.

In the five experiments presented in Table IV the fluxes of chloride into the blood (M_1) and into the lumen (M_0) are given. Since net chloride movement was from the lumen to the blood, the ratio $M_1:M_0$ is greater than 1.0.

In Table V the unidirectional sodium fluxes are

TABLE V

Sodium flux ratios

Expt.	No. of one hour flux periods	$\frac{[Na^+_{plasma}]}{[Na^+_{lumen}]}$	$\frac{M_1}{M_0}$*	Observed† ratio	Calculated† ratio	$\frac{\text{Observed}}{\text{Calculated}} \times 100$
						%
1	5	$\frac{143}{135}$	$\frac{81}{24}$	3.3	0.55	600
2	6	$\frac{150}{135}$	$\frac{93}{34}$	2.7	0.40	670
3	6	$\frac{145}{141}$	$\frac{55}{26}$	2.1	0.50	420
4‡	6	$\frac{146}{138}$	$\frac{30.7}{38.7}$	0.80	0.40	200
5‡	4	$\frac{150}{142}$	$\frac{40}{63.8}$	0.64	0.42	147

* M_1 is influx (lumen to blood); M_0 is outflux (blood to lumen). Flux expressed as μEq. per Gm. per hour.

† Observed ratio for sodium calculated from $\frac{M_1}{M_0} \cdot \frac{[Na^+_{plasma}]}{[Na^+_{lumen}]}$; calculated ratio from 1/antilog $\frac{E(mV.)}{60}$.

‡ Experiments 4 and 5 characterized by net secretion of water into the lumen, Experiments 1, 2 and 3 by net absorption.

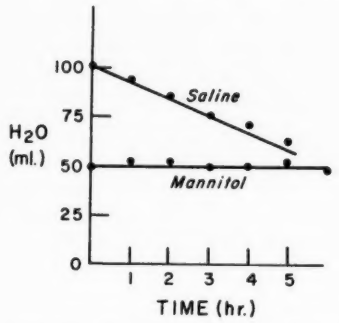

FIG. 1. VOLUME OF PERFUSATE AT EACH SAMPLING HOUR IS PLOTTED AGAINST TIME

See text.

presented. In the first three experiments there was a net transfer of sodium from lumen to blood and the ratio of sodium influx (M_i) to the sodium outflux (M_o) is greater than 1.0. The ratio of sodium fluxes was less than 1.0 in the remaining two experiments since there was net sodium movement into the lumen.

For electroneutrality the sum of the charges of the ions being transferred must equal zero. This was not observed in each sampling period due to slight errors in the chemical results used in calculating net transfer, but the average of the net transfer of ions in each experiment, as well as the mean for all experiments, was found to approach zero.

Water

The data from two experiments presented in Figure 1 show that there was no significant absorption of water without net absorption of solute. In one a saline solution was recirculated through the lumen and in the other isotonic mannitol was used. Changes in water content were quite small in the latter experiment because of the slight absorption of mannitol. However, in the two experiments in which there was extensive bicarbonate secretion, net water transfer was quite variable as indicated by the experiment represented in Table III. The osmolality of the luminal contents remained essentially constant, indicating that absorption or secretion was isotonic.

DISCUSSION

The preceding results confirm the previous observations on the isolated large intestine which showed that sodium was absorbed against its electrical and chemical gradient (4, 5). This net transfer requires energy and can be defined as active. In two experiments the secretion of bicarbonate was observed to be against its electrical and chemical gradient; this secretion also must be active. Since chloride absorption and potassium secretion were observed to be in the direction of their electrical gradients and opposite to their chemical gradients additional information is necessary to describe their means of transfer more fully.

Ussing (11) has developed the following equation to describe the passive movement of an ion across a semi-permeable membrane:

$$\frac{M_i}{M_o} = \frac{a_o}{a_i} \cdot e^{(zF/RT) \cdot E} \qquad 5)$$

or

$$\log \frac{M_i \cdot a_i}{M_o \cdot a_o} = \frac{zF}{RT} \cdot E = \frac{E(mV.)}{60} \cdot \text{at } 37^\circ \text{ C.}$$

where M_i and M_o are the influx and outflux of the ion; a_i and a_o, respectively, the chemical activity of the ion in plasma and lumen; z, F, R, T., respectively, the charge of the ion, the Faraday, the gas constant and absolute temperature; and E(mV.), the electrical potential difference across the membrane in question.

The relationship between passive ionic transfer and electrical and chemical potential gradients is evident from Equation 5. Any significant deviation from this equation must be explained in terms of an energy expenditure or other factors not considered. One of these factors, "solvent drag," will be discussed with relation to the transfer of chloride.

Since sodium is actively transported, the ratio of its unidirectional fluxes does not follow the above equation. However, as noted in two experiments (Table V, Experiments 4 and 5), net sodium movement was from a higher to a lower electrochemical potential and accompanied bicarbonate and water into the lumen. At the observed potential the observed flux ratio for sodium is larger than the calculated value. One possible explanation for this finding is that sodium is still being actively transported even though this "active transport" is being overwhelmed by the other forces acting on the sodium ion.

Flux rates of sodium across the isolated large intestine of the frog have been expressed as μEq. per unit area (12), but recalculation of values on

a weight basis gave net sodium fluxes of 30 to 60 μEq. per Gm. of tissue per hour. Thus the rate of sodium absorption across the isolated frog colon at 25° C. is of the same order of magnitude as the rate of sodium absorption in the dog.

Chloride is transferred from a higher to a lower electrochemical potential. The electrical potential difference between lumen and blood is the major driving force. With net water secretion into the lumen there is still net chloride transfer from lumen to blood (Table III). In comparing the dog and frog colon with respect to the transepithelial fluxes of chloride the following differences occur: One, the net chloride flux during absorption in the dog (Table I) is greater than the net flux of 10 to 20 μEq. per Gm. of tissue per hour in the frog. Two, unlike the observations on the frog *in vitro*, the data presented here do not suggest appreciable exchange diffusion of chloride.

In Table IV the calculated and observed ratios of chloride are compared in five experiments, and they do deviate from the values calculated for passive diffusion. These deviations could be explained by the following factors: *1*) Although chloride is transferred predominately by passive diffusion there may be a component of active chloride transport or exchange diffusion.[1] *2*) In the presence of rhythmical potential variations the potential difference as determined by planimetry may not be the true mean potential. *3*) It is likely that the last factor, "solvent drag," is the most important for the following reasons:

"Solvent drag" is the physical effect of net water flow exerted upon solute and is a function of membrane structure as defined by Ussing and Andersen (4). This flow reduces solute movement in the opposite direction, while enhancing it in the same direction. Net water flow into the blood (Table IV, Experiments 1 through 3) would increase chloride influx and/or decrease outflux. Net water flow into the lumen would have the opposite effect (Table IV, Experiments 4 and 5). Such an effect was observed on the chloride flux ratios: The chloride ratio is lower than predicted by Equation *5* when net water secretion occurs and higher than predicted when net water absorption takes place. On the other hand, the observed sodium flux ratio (Table V, Experiments 4 and 5) is larger than the calculated value for passive diffusion despite net water transfer into the lumen. This indicates that active sodium absorption was still operative during net sodium transfer into the intestine.

The limitations of Equation *5* as presented here for the *in vivo* intestine have been predicted by Ussing and he has developed an equation which takes "solvent drag" into account (13). A rough estimate of the "solvent drag" force can be obtained from the flux ratio of water which was not measured. However, Berger has recently measured D_2O fluxes across the colonic mucosa of the unanesthetized dog (14). In experiments characterized by net water absorption, the water flux ratios were 1.23 to 1.32. If one uses this figure to recalculate the chloride ratios (Table IV, Experiments 1 through 3) there is a closer agreement between the observed and calculated results. This suggests that chloride transfer is influenced by "solvent drag" as well as by the electrochemical gradient.

The net flux of potassium from blood to lumen was in the proper direction for passive transfer (Tables I through III). This does not rule out an active secretion of potassium, or a sodium-potassium exchange at one surface of the epethelial cell, a mechanism postulated for the kidney and frog skin (15, 16). The unidirectional transfers of potassium were not measured and the nature of the experiment did not permit the attainment of the steady state where $M_1 = M_0$ in order to test the relationship:

$$E = 60 \log \frac{K^+_{\text{lumen}}}{K^+_{\text{plasma}}}.$$

Subsequent studies have revealed that, in some instances, potassium secretion may be active, and this will be the subject of a forthcoming report.

In all but two experiments there was net absorption of bicarbonate along its electrical gradient. Although the original perfusate contained 6 times as much chloride as bicarbonate, up to 20 times as much chloride was reabsorbed in 11 experiments. Thus despite net absorption of bicarbonate, the concentration of bicarbonate in the

[1] Chloride transfer across the isolated large intestine of the frog during absorption involves a component of exchange diffusion. If exchange diffusion were operative in the dog the chloride flux ratios would be significantly less than and not greater than the values calculated for passive diffusion (12).

lumen increased. This suggests that the epithelium of the large intestine is less permeable to bicarbonate than chloride, or there is active secretion of bicarbonate into the lumen. The latter mechanism can occur, as shown in Table III, where bicarbonate secretion into the lumen was opposite to its electrochemical gradient and was accompanied by water, sodium and potassium. The ratio of bicarbonate to water in the net secretion was 130 mEq. per L. in these latter instances. This is not unreasonable when compared to pancreatic secretions containing 110 mEq. per L. of bicarbonate or more, and gastric secretions as high as 150 mEq. per L. of hydrogen ions. The variations in active bicarbonate secretion may be due to changes in neurohumoral influences, since stimulation of the pelvic nerves and administration of cholinergic drugs induced alkaline secretion by the colon (6).

In this discussion the term bicarbonate secretion has been used for brevity. However, these experiments do not distinguish among bicarbonate secretion, hydroxyl ion secretion or hydrogen ion absorption. Previous studies on the isolated frog large intestine suggested that in addition to sodium there was another ion being actively transported, although this ion was not identified (12). If active bicarbonate secretion is under the influence of a neurohumoral mechanism it is not surprising that it could not be demonstrated in the *in vitro* system, but was clearly demonstrated and found to have considerable variability *in vivo*.

Driving forces for net water flow are the gradients of hydrostatic and osmotic pressure. The latter is created by the two ion transport mechanisms: sodium absorption and bicarbonate secretion. It was noted that there was no significant net transfer of water without solute. In addition to electrical and chemical forces, this net water movement can be a third driving force for other diffusing solutes (11). The effect of this "solvent drag" has been discussed previously with regard to the transfer of chloride.

In the preceding discussion the assumption has been made that the measured potential difference represents the transmucosal potential. This assumption seems justified in view of the following observations: First, the isolated large intestine when stripped of its outer muscular coat can generate a potential difference and actively transport sodium (4). Second, Rehm has presented evidence that no significant potential difference can be measured across the muscularis and peritoneal surface of the stomach in the presence of a significant electromotive force across the stomach wall (17). Third, the peritoneal cavity and circulating venous blood were found to be equipotential in the present study.

Transepithelial potential differences have been considered to be due directly to the unidirectional active transport of an ion (1), *e.g.*, sodium absorption and/or bicarbonate secretion. However, recent studies indicate that such bioelectrical potentials may be diffusion potentials resulting from active ion-exchange mechanisms involved in active transport (16). It is interesting that strophanthidin, a known inhibitor of cation transport (18), causes a drop of at least 50 per cent in the observed potential and partial inhibition of the sodium and bicarbonate active transport mechanisms across the large intestinal epithelium (19).

SUMMARY

An electrical potential difference of 10 to 40 mV. (the lumen being negative with respect to the blood) was measured across the large intestine of 13 anesthetized dogs. Correlation of this potential difference with chemical events demonstrated two active transport mechanisms: sodium absorption and bicarbonate secretion. Net water flow occurred, and its direction and magnitude were dependent on the degree of active transport of sodium and/or bicarbonate. The absorption of chloride was in the direction of its electrochemical potential gradient, probably modified by net water movement. Potassium was transferred from the blood to the lumen, in the direction of its electrical gradient but opposite to its chemical gradient.

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THE FATTY ACIDS OF HUMAN MILK. II. ALTERATIONS PRODUCED BY MANIPULATION OF CALORIC BALANCE AND EXCHANGE OF DIETARY FATS* †

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The observation that human serum lipid concentrations may be altered by diet and the concept that this may be due to the nature of the dietary fatty acids (1) has intensified interest in the study of the metabolism of individual fatty acids. Detailed studies of natural lipid mixtures and their fatty acid components are now made possible on a microscale by recently developed methods of lipid analysis (2, 3). However, in clinical studies many organs of prime metabolic interest are not accessible for examination. In this circumstance the study of human milk fat provides unique advantages since it is a metabolic end-product which may be sampled frequently without risk or discomfort to the patient. It is a simple mixture of lipids composed almost entirely of triglycerides; however, we have noted at least forty different fatty acids in milks of mothers on *ad libitum* diets (4).

Purposeful alterations of human milk fat by dietary means was first attempted by Thiemich in 1898 (5); he showed marked changes in the iodine number of the milk fat after feeding mothers unsaturated fat. Recent studies by Söderhjelm (6) have shown an increased content of diene fatty acids following feedings of corn oil and of higher polyene acids following feedings of fish oil.

The present report describes specific alterations of the fatty acid pattern of human milk produced by rigidly controlled variations in the maternal diet. By observing the effects of dietary conditions which might favor *de novo* synthesis of fatty acids in the mammary gland or, alternatively, transfer from storage depots or from dietary fat, it was hoped that some insight might be gained into fundamental mechanisms of fat metabolism in organs other than the breast. These objectives were partially achieved, but certain data indicated that fatty acid synthesis in the human breast is somewhat specialized.

METHODS

Nomenclature. The very large number of acids of different chain lengths and double-bond contents which are now demonstrable by gas-liquid chromatography necessitates a shorthand designation. In this report fatty acids are numbered according to chain length (number before colon) and double-bond content (number after colon). Thus, 18:0 refers to the normal saturated C_{18} acid, stearic; 18:2 = C_{18} diene, and so forth.[1] Wherever definitive information on double-bond structures is available, it would be more appropriate to specify the exact structure, *i.e.*, octadeca-9,12-dienoic acid (or linoleic acid), but when this is lacking, acids are called dienes, trienes, and so on. The weight percentage compositions of fatty acid mixtures are called *fatty acid patterns.*

Metabolic ward procedure. The subject of this study was a 23 year old white para III whose pregnancy was normal with spontaneous delivery at term.[2] She had mild chronic spastic colitis limited to the rectosigmoid colon for one year. The patient and her normal newborn daughter were admitted to this hospital on the fifth postpartum day and remained in the rooming-in unit of a metabolic ward for seven weeks' study. This metabolic ward has been described in detail elsewhere (1).

The mother was ambulatory but sedentary. The baby was exclusively breast fed, six feedings per day at four hour intervals. Both were weighed daily under standard conditions. Feedings were successively initiated at alternate breasts, and both were offered at each nursing. The mother was nourished solely by an orally administered liquid formula, the total daily intake being divided into five equal portions, supplemented with vitamins and minerals as previously described (7). Water was offered *ad libitum.* The mother's intakes exceeded

* A preliminary report of this study was presented on July 17, 1957, at the Fourth International Conference on Biochemical Problems of Lipids, Oxford, England.

† Supported in part by grants from the Nutrition Foundation and the United States Public Health Service (H-2539).

‡ Visiting investigator. Present address: National Institute for Medical Research, London, England.

[1] This system of designation was evolved in discussions with Dr. V. P. Dole.

[2] The patient was referred through the cooperation of the Department of Obstetrics, Lincoln Hospital, Bronx, N. Y.

recommended standards for nursing mothers (8). This regimen has proven adequate for growth, maintenance and physical activity in long-term clinical feeding studies carried out over the last five years (1).

Maternal milk output was determined by weighing the baby immediately before and after each nursing. At the end of each dietary period, milk was collected and sampled during a 24 hour period as previously described (4), 90 per cent of the milk being fed to the infant by bottle. Additional samples of four hour production were obtained every other day in similar manner. Milks were refrigerated immediately after collection and samples were frozen for storage. Fasting blood specimens were taken during each 24 hour milk collection.

Chemical methods. Milk lipids were quantitatively extracted with 95 per cent ethanol-ethyl ether (3:1), and the solvent-free residue of the extract was dissolved in petrol ether (30 to 60°) to obtain a lipid extract free of nonlipid contaminants. This method has been validated elsewhere (4). More than 98 per cent of the milk lipid was triglyceride, with only small amounts of free fatty acids and traces of cholesterol and phospholipids. Specific analyses of short-chain acids (C_{1-6}) have not been carried out; however, other data (4) indicate that in human milk these acids must comprise less than 4 per cent of total acids. For present purposes the milk lipid was considered to be entirely triglyceride and was not fractionated. Methyl esters of the fatty acids were formed as described previously (4).

Analyses of the composition of the complex mixture of fatty acids (fatty acid patterns) were made by two independent but complementary methods, gas-liquid chromatography and ultraviolet spectrophotometry after isomerization with alkali. The latter method (9) offered a quantitative picture of the five polyene groups (diene through hexene) without regard to fatty acid chain length, and was based on use of constants derived from study of certain pure C_{18-22} acids. As applied in this report, gas-liquid chromatography (2) partially described and measured acids of chain length C_{6-18}, in a few cases through C_{20}. The eight major acids, comprising more than 95 per cent of total C_{6-18} acids, are named with considerable assurance, and all except the linoleate and linolenate pair (18:2 + 3) were clearly separated. For resolution of this pair, the data obtained by ultra-violet spectroscopy have proven to be completely reliable (4).

Gas-liquid chromatography of the mixed methyl esters was carried out at 197° with Apiezon M® as the stationary phase and nitrogen as the mobile phase. The gas-density balance was used as detector (10), and records were analyzed by triangle mensuration with the calibration factors of James and Wheatley (11). The column efficiency was 2,900 theoretical plates, calculated at methyl stearate. With the separation factor of stearate/oleate = 1.18, these esters were 99 per cent separated. In another report describing these techniques in detail (12) we have shown the remarkable stability of highly unsaturated long-chain fatty acid esters on such columns.

Our most recent studies have shown the presence in human milk of a wide array of C_{19-22} acids, many as yet unidentified (4). However, these acids comprise only about 4 per cent of the total acids, and the conclusions to be drawn from the present data, which are restricted to consideration of the C_{6-18} acids, seem in no way weakened by the omission of $C_{>18}$ data.

Serum lipid classes (triglycerides, free and total cholesterol, and phospholipids) were analyzed as described elsewhere (13). Cholesterol esters, triglycerides and total phospholipids were isolated by chromatography on silicic acid (3). After preparation of the methyl esters (14) analyses of the fatty acids in each of these ester groups were made by gas-liquid chromatography as described above.

Experimental design. In this study the mammary gland is regarded as a metabolic unit from which milk is derived as the net result of the gland's metabolic activity; possible differences in function of the anatomic divisions of the gland are disregarded. Three possible sources of milk lipids were considered: synthesis *in situ*, transfer via the blood stream from fat depots and other extramammary sites of fat synthesis, and transfer of dietary fat. It seemed reasonable to postulate that the interplay of forces acting to favor one or another source of milk fat would be influenced by the total energy balance of the mother and also by the proportion of fat and nonfat calories in her diet. By defining the milk fatty acid pattern when these experimental variables were rigidly controlled, it was hoped that the source of the milk fat could be identified.

The experimental study was divided into seven consecutive feeding periods (Table I). Each period was at least four days in duration, a time sufficient for the dietary fat to exert a maximal effect on the milk fat (5, 6).

TABLE I

Experimental design *

	Dietary period						
	I	II	III	IV	V	VI	VII
Caloric intake	*ad lib.*	2,750	3,750	1,838	3,750	2,938	2,938
Fat calories, % of total		40%	~0%	~0%	70%	70%	40%
Fat intake, Gm./day		123	3.0	1.4	292	228	131
Type of fat		Lard	Cow's milk	Cow's milk	Corn oil	Corn oil	Corn oil
Days postpartum	5–11	12–18	19–26	27–33	34–43	44–48	49–52

* All illustrations show this scheme in abbreviated form.

In Period I the patient was fed a regular hospital diet *ad libitum* until lactation was established. Thereafter, liquid formula feeding was used exclusively. The entire protein intake was derived from cow's milk protein at levels of 15 per cent of total calories in all six formula periods. Changes in the proportion of fat calories were effected by reciprocal changes in carbohydrate calories, and, in addition, two dietary fats were interchanged which were readily recognizable by their fatty acid compositions (lard and corn oil).

In Period II lard contributed 40 per cent of total calories. Since pig depot fat is very similar in fatty acid composition to the fat mixture in an average American diet (1, 15), this period served to mimic Period I. The total daily caloric requirement for maintenance of body weight was established at 2,750 calories. In Period III a "fat-free" diet was fed. In order to promote fat synthesis from carbohydrate at all sites (including the mammary gland) and to minimize the transport of fat out of storage, excess calories were fed (1,000 calories *above* the maintenance requirement) in the form of carbohydrate and protein. Traces of dietary fat shown in Table I were carried into the formula in the cow's milk protein. In Period IV, in order to stimulate mobilization of depot fat and its utilization by the breast, 1,000 calories *less* than the maintenance requirement were fed. Again, the "fat-free" formula was fed in order that the depot fat pattern might be identified in the milk fatty acids.

In Period V a corn oil formula was fed. To favor transfer of this diet fat directly into milk fat and to suppress synthesis and transport of endogenous fat, 1,000 excess calories were fed with 70 per cent of total calories derived from corn oil. During Periods VI and VII the total caloric intake was reduced to that required to maintain body weight constant (2,938 calories per day at this time), and the proportion of corn oil calories was successively 70 and 40 per cent. These two final periods were designed to determine the effect of fat to carbohydrate ratios on the transport of dietary fat into milk fat, as well as for sake of comparison with Period II.

To summarize the experimental design, endogenous synthesis of milk fat from carbohydrate was encouraged by feeding excess calories and no fat (Period III); transfer of depot fat was promoted by feeding deficient calories (Period IV); and transfer of dietary fat by feeding excess calories as corn oil (Period V). Effects of substituting one fat for another were compared in Periods II (lard) and VII (corn oil).

RESULTS

Lactation

Lactation was readily established by the ninth postpartum day, with a daily milk production thereafter averaging 723 ± 65 Gm. during Periods II through VII (Figure 1). More milk was regularly obtained when 24 hour collections were made by mechanical and manual expression than when the breasts were nursed by the baby. The concentration of milk fat in the 24 hour collections averaged 3.5 Gm. per 100 ml. milk to give a total fat production of 24 to 30 Gm. per day (Figure 2). The two breasts produced almost equal amounts of milk fat after lactation was well established. Neither the daily milk volume nor

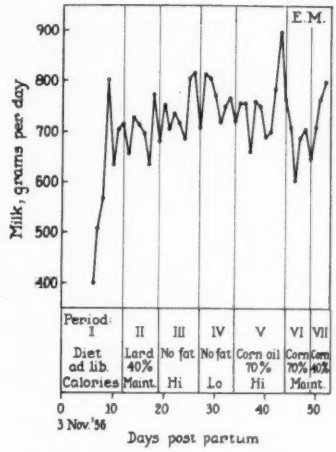

FIG. 1. DAILY MILK PRODUCTION

Total daily milk production was not significantly changed during the various dietary periods.

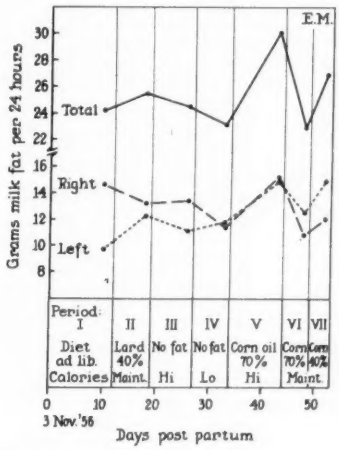

FIG. 2. DAILY MILK FAT PRODUCTION

Total daily milk fat production was highest on a diet high in both calories and fat. There was no significant difference in the milk fat production of the two sides during the experimental period.

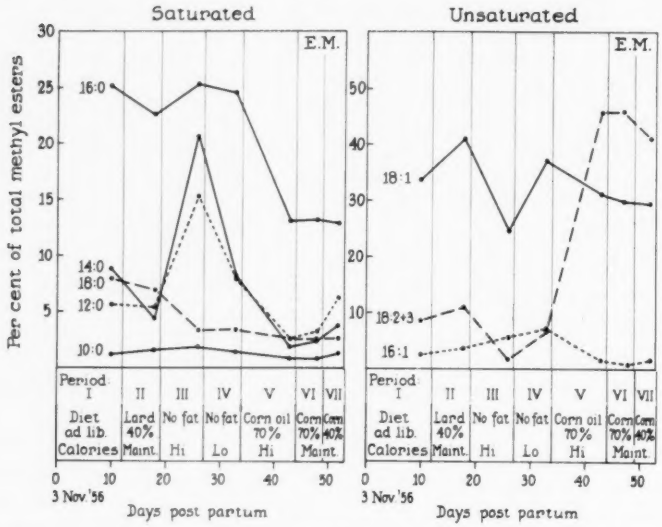

FIG. 3. THE PROPORTION OF MAJOR FATTY ACIDS IN MILK FROM THE LEFT BREAST PER 24 HOURS, AS DETERMINED BY GAS-LIQUID CHROMATOGRAPHY

There are striking changes in both saturated and unsaturated acids during various feeding periods.

daily fat production appeared to be influenced by changes in the mother's diet. The baby remained healthy and gained weight constantly at an average rate of 28.3 Gm. per day during the period of hospitalization, and on this basis lactation was judged to be normal. The mother's sense of well being was unimpaired throughout, and complaints attributed to mild chronic colitis remained unchanged.

Milk fatty acid patterns

The proportions of the major fatty acids in the 24 hour milks from the left breast are shown in Figure 3. Milk samples obtained simultaneously from right and left breasts uniformly had almost identical patterns, whether analyzed by gas-liquid chromatography or by ultraviolet spectroscopy after alkali isomerization. Moreover, in each feeding period analyses of terminal 24 hour col-

TABLE II

Comparison of fatty acid patterns in dietary fat and milk lipids *

Diets	Period II Lard (40% of total calories), maintenance calories		Period IV "Fat-free" deficient calories		Period V Corn oil (70% of total calories), excess calories		Period VII Corn oil (40% of total calories), maintenance calories	
Fatty acids	Lard	Milk	Depot fat†	Milk	Corn oil	Milk	Corn oil	Milk
12 : 0	0.2	4.6	0.1	7.9	0.1	3.0	0.1	4.9
14 : 0	1.7	9.3	2.7	9.0	0.3	2.3	0.3	4.3
16 : 0	22.3	23.8	24.0	23.5	12.6	13.1	12.6	12.7
16 : 1	2.7	3.1	5.0	6.8	0	1.4	0	1.5
18 : 0	9.3	7.8	8.4	3.2	2.7	3.0	2.7	2.9
18 : 1	44.9	42.6	46.9	36.9	30.4	31.4	30.4	28.7
18 : 2+3	13.5	10.3	10.2	7.3	52.9	43.0	52.9	42.0
% of total acids	94.6	96.5	97.3	94.6	99.0	97.2	99.0	97.0

* Data calculated as weight percentages of total methyl esters.
† Fifty-three year old female, Cramer and Brown (16).

lections were closely mimicked by those of milks obtained one or two days previously, indicating that by two days after each dietary change the maximal dietary effect had been obtained.

Figure 3 demonstrates that palmitic (16:0) and oleic (18:1) acids predominated in milk fat during Periods I and II, when fat calories were 40 per cent, total calories adequate to maintain body weight, and the dietary fat pattern that of lard or the average American diet (15). The proportions of all acids in the milk samples during these two periods closely resembled those seen in the milk of mothers on *ad libitum* diets (4).

In Period III the weight percentages of 12:0 and 14:0 doubled at the expense of 18:0, 18:1 and 18:2 + 3. On a *molar* basis 12:0 plus 14:0 made up 39 per cent of the total acids. The proportions of 16:0 and 16:1 remained essentially unchanged. Thus, when endogenous synthesis of fatty acids from carbohydrate precursors was promoted by feeding excess calories and a "fat-free" diet, saturated acids of intermediate chain length appeared in the milk in striking proportions.

In Period IV the fatty acid pattern of the milk fat came to resemble that found when the 40 per cent lard diet was fed (Period II) or during *ad libitum* feeding (Period I), and furthermore it resembled that reported (16) for human depot fat (Table II). Thus, when deficient calories of

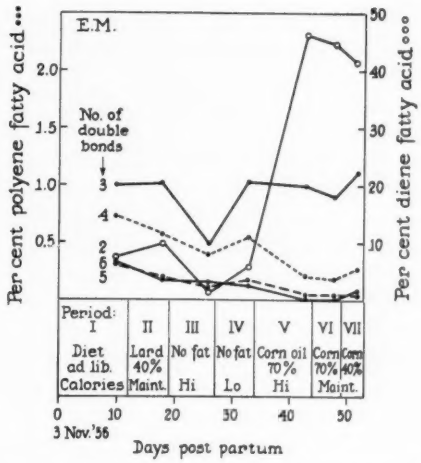

FIG. 4. THE POLYENE FATTY ACIDS IN 24 HOUR MILK COLLECTIONS FROM THE LEFT BREAST

Note that the diene scale (on the right) is 20 times that of the other polyenes.

TABLE III

Polyene contents of dietary and milk fats expressed as percentages of total fatty acids by weight

Period	Breast	Dienes		Trienes		Tetraenes		Pentaenes		Hexaenes	
		Diet	Milk	Diet	Milk	Diet	Milk	Diet	Milk	Diet	Milk
I	L*		7.6		1.0		0.7		0.3		0.3
Ad lib.	R†		7.8		1.0		0.7		0.3		0.4
II	L	12.0	9.9	0.8	1.0	0.3	0.6	0.1	0.2	tr	0.2
40% lard	R	12.0	11.8	0.8	1.4	0.3	0.6	0.1	0.2	tr	0.2
III	L		1.2		0.5		0.4		0.1		0.1
Fat-free, excess calories	R		1.5		0.5		0.4		0.1		0.1
IV	L		5.9		1.0		0.6		0.2		0.1
Fat-free, deficient calories	R		6.4		1.1		0.6		0.2		0.1
V	L	50.9	46.1	1.6	1.0	0.7	0.2	tr‡	tr	tr	tr
70% corn, excess calories	R	50.9	43.3	1.6	0.9	0.7	0.2	tr	tr	tr	tr
VI	L	50.9	44.5	1.6	0.9	0.7	0.2	tr	tr	tr	tr
70% corn, maint. calories	R	50.9	44.7	1.6	0.9	0.7	0.3	tr	tr	tr	tr
VII	L	50.9	41.4	1.6	1.2	0.7	0.3	tr	tr	tr	tr
40% corn, maint. calories	R	50.9	41.4	1.6	1.1	0.7	0.3	tr	tr	tr	tr

* Left breast.
† Right breast
‡ Tr = <0.1 per cent.

a "fat-free" formula were fed, it appeared that depot fat was mobilized and transferred into the milk.

In Periods V through VII when corn oil was the sole dietary fat, the content of 18:2 + 3 increased more than fivefold over all previous levels. This increase occurred mainly at the expense of the saturated acids. Thus, the major acid of corn oil (18:2) appeared in breast milk in large amounts, and the pattern of milk acids approached that of corn oil itself (Table II). In Periods VI and VII, when the percentage of corn oil calories was reduced from 70 to 40 per cent of total calories, 18:2 + 3 decreased slightly, while 12:0 and 14:0 increased.

Table II demonstrates that milk fat is distinctive in having a higher content of 12:0 and 14:0 acids than the dietary fat in all feeding periods. In fact, these contents of lauric and myristic acids, noted also in the milks of mothers on *ad libitum* diets (4), are higher than those encountered in most other animal fats except butter oil.

Not shown in tabular form, the odd-numbered acids, C_{7-17}, occurred in amounts less than 1 per cent and were depressed during Periods II, V and VI. C_{13} and C_{15} acids were not affected in Period III when 12:0 and 14:0 increased so strikingly.

The polyene classes measured by ultraviolet spectroscopy are shown in Figure 4. The diene curve clearly resembles that of 18:2 + 3 in Figure 3, indicating that the 18:2 + 3 of Figure 3 and Table II refer almost entirely to 18:2. This is borne out by the data in Table III, which indicates that trienes at no time constituted more than 1.4 per cent of total fatty acids. In addition, the ultraviolet data indicated that the tri- to hexaenoic acids never exceeded 2.5 per cent of total acids. Thus, it is clear that the very highly unsaturated C_{19-22} acids which were *not* analyzed in these chromatographic runs were low in concentration.

Figure 4 shows also that all polyenes decreased in Period III when 12:0 and 14:0 increased strikingly. In Periods V through VII, when dienes were highest, tetraenes did not increase.

Serum lipids and fatty acid patterns

The trends in concentrations of the mother's serum lipids were as expected (Figure 5). Cholesterol and phospholipid concentrations were lowest when corn oil was fed and were somewhat higher on the lard and "fat-free" diets. The triglycerides increased sharply when the "fat-free" diet in caloric excess was fed in Period III. However, all feeding periods were too short to permit establishment of the full effect of each diet on serum lipid concentrations. Previous long-term studies (1) have indicated that two or more weeks are usually required before a new equilibrium is established.

The fatty acids of the major ester groups in the serum of the mother were determined on three occasions at times of 24 hour milk collections: in Periods II, III and V and VI (pooled). The data in Periods II and V and VI indicated that the dietary fatty acid pattern was most closely simulated by the pattern of the triglyceride fatty acids. In addition, the cholesterol esters showed considerable avidity for the 18:2 of corn oil. The

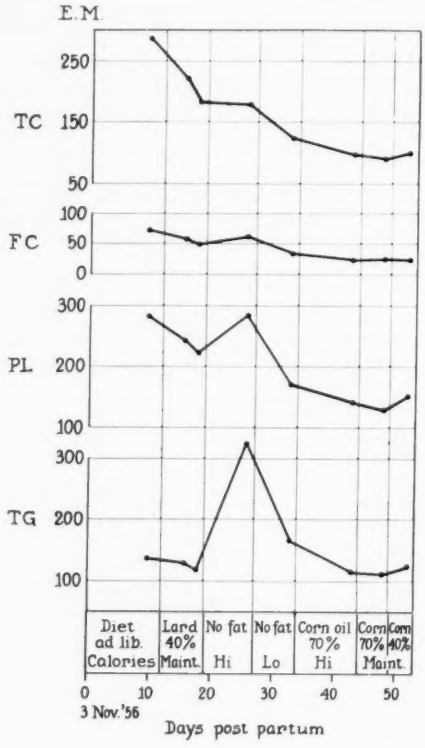

FIG. 5. SERUM LIPID CONCENTRATIONS DURING EACH FEEDING PERIOD

From top to bottom: total cholesterol, free cholesterol, phospholipids and triglycerides, all given in mg. per cent.

phospholipids were least sensitive to shifts in dietary fats, and in this group saturated acids comprised 55 to 70 per cent of total acids with a 16:0 per 18:0 ratio of nearly two. There was some variation in content of 18:1 and 18:2 + 3 in the phospholipids, but these changes were less marked than in the other two ester groups.

The main usefulness of these analyses was shown in Period III, when the milk lipids contained a marked increase in 12:0 and 14:0 acids. By contrast, none of the serum ester groups contained larger than usual amounts of these two acids. This disparity in serum and milk contents of 12:0 and 14:0 has been seen subsequently in three other patients ingesting fat-free diets.

The possibility remains that comparison of patterns of fatty acids in the serum ester groups with that in the milk can indicate which ester group in the serum most effectively transfers its fatty acids into the milk. The present data suggest that the triglycerides serve this function, but this aspect of the problem remains to be explored in greater detail.

DISCUSSION

The experimental results confirm our working hypothesis that the composition of milk fat can be altered by changes in total caloric intake as well as by changes in types of dietary fats. In agreement with previous workers (5, 6) the data indicate clearly that dietary fatty acids are readily transported into milk fat. The marked changes in milk fatty acid patterns which were produced did not affect total daily milk or milk fat output.

The importance of positive and negative energy balance states in determining the character of milk fat has not been noted previously in animals or in man. With deficient caloric intake, milk fat closely resembled human depot fat (and indeed lard also), suggesting a transfer process. On the other hand, when excess calories of a "fat-free" diet were fed, we expected carbohydrate to be converted primarily into oleic, palmitic and stearic acids in the various sites of synthesis (including the breast), since the fats of animals grown on fat-poor diets (17) are composed mainly of these three acids (75 per cent). However, in this dietary situation 60 per cent of the fatty acids in breast milk were lauric, myristic and palmitic acids, while stearic, oleic and linoleic acids actually decreased. Since none of the serum ester groups showed high concentrations of lauric and myristic acids, there was no evidence that these acids, if synthesized in an extramammary site, were transported to the breast via the blood.

While it is possible that these acids may find their way into breast milk by selective rapid transfer from the small amounts present in one of the serum ester groups or from the nonesterified fatty acid fraction in serum (18, 19) we are tempted to suggest two other mechanisms. Figure 3 shows that the major unsaturated acids decreased in Period III during positive energy balance on a fat-free diet. If the mammary gland had a low capacity for synthesis of unsaturated acids, the mixture of saturated acids formed from carbohydrate would have an exceedingly high melting point, *unless* a considerable amount of intermediate chain-length acids was present. When hogs are raised on low-fat rations, their depot fat contains a high concentration of oleic acid as well as palmitic and stearic acids. The net effect is a triglyceride which melts below body temperature. However, if the human breast differed from adipose tissue in having a deficiency of enzymes which synthesize unsaturated acids, the milk glycerides would have a high melting point if 12:0 and 14:0 were not present in significant concentrations. Thus, the increased amounts of these acids in the milk in Period III may represent an adaptive response to an unusual dietary challenge.

Alternatively, the human breast may be deficient in the enzymes which add acetate fragments after fatty acids have reached C_{14} and C_{16} in chain length, since Figure 3 shows that all C_{18} acids decreased in Period III. In their studies of lactation, Folley and French (20) demonstrated that ruminants synthesize fatty acids from acetate while non-ruminants utilize glucose. Popjak, French, Hunter and Martin (21) extended these findings in goats to show a remarkable cut-off of synthesis beyond C_{16}. However, the postulate that the human breast lacks the enzymes which carry synthesis past C_{14} seems weakened by the continually high concentration of 16:0 in Period III.

A definitive choice between the two hypotheses cannot be made on the basis of present data. Indeed, it cannot be forgotten that some transfer of acids from the blood may be taking place and may contribute to the content of 18:1 and 16:0 in the milk during Period III. Even though

none of these questions can be resolved with currently available facts, it seems apparent that fatty acid metabolism in the human mammary gland is not identical to that in adipose tissue.

CONCLUSIONS

The fatty acid composition of human breast milk can be radically altered without affecting milk volume or milk fat output. During energy equilibrium milk fat closely resembles dietary fat, but, when deficient calories are fed, milk fat approaches the composition of human depot fat. These findings suggest that, under either of these nutritional conditions, transfer of dietary or depot fat dictates the fatty acid pattern of human breast milk.

However, when excess nonfat calories are fed to the mother, the milk shows a striking increase in lauric and myristic acids and a marked decline of all polyenoic acids. Yet, none of the serum ester groups (cholesterol esters, triglycerides and phospholipids) show a parallel rise in these acids. Thus, synthesis of fatty acids in the breast appears to be promoted when excess calories are fed, and the accumulation of C_{12} and C_{14} acids in the milk suggests that human mammary fatty acid synthesis differs in important respects from that in extramammary depots.

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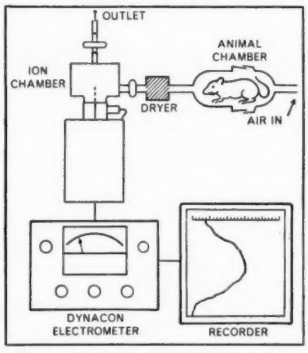

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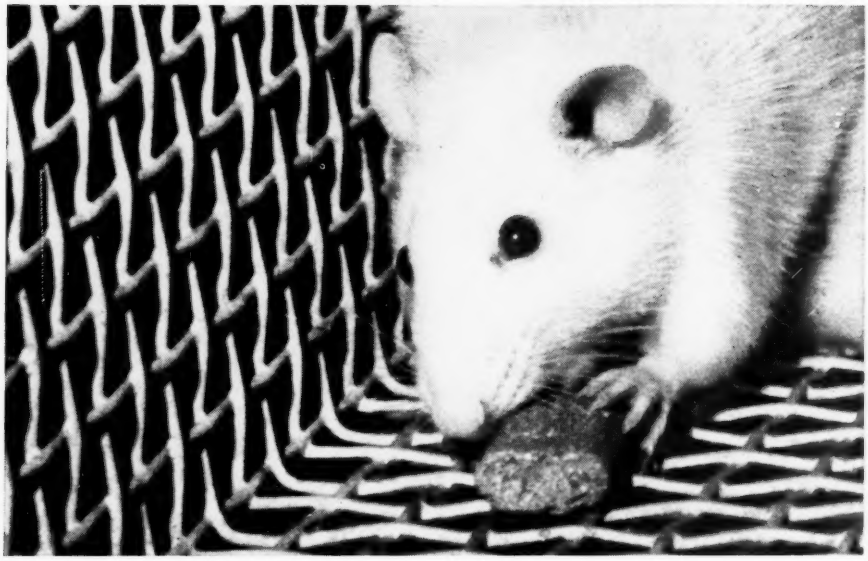

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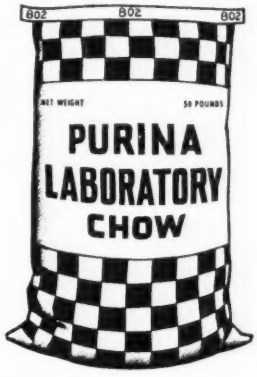

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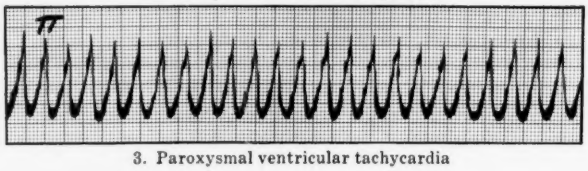

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